Entwicklung und Evaluierung einer Multiparametertechnologie zur serologischen Diagnostik von Autoimmunerkrankungen

von der Fakultät für Umwelt und Naturwissenschaften der Brandenburgischen Technischen Universität Cottbus–Senftenberg genehmigte Dissertation zur Erlangung des akademischen Grades eines Dr. rer. nat.

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Abkürzungsverzeichnis

ANCA assoziierte systemische Vaskulitis	AAV
Anti-neutrophile z(c)ytoplasmatische Antikörper	ANCA
Anti-nukleäre Antikörper	ANA
Atypische ANCA	aANCA
Autoantikörper	AAk
Autoimmundiagnostik	AID
Autoimmunerkrankungen	AIE
Autoimmunhepatitis	AIH
Centromer-B Protein	CENP-B
Crithidia luciliae Fluoreszenz Test	CLIFT
Colitis Ulcerosa	UC
Deamidiertes Gliadin	DeaGlia
Doppelsträngige Desoxyribonukleinsäure	dsDNS
Einzeldomänen-Antikörpern	VHH
Entzündliche Darmerkrankung	IBD
Enzyme-linked Immunosorbent Assay	ELISA
1-ethyl-3-(3-dimethylaminopropyl)carbodiimid-Hydrochlorid	EDC
Fluoresceinisothiocyanat	FITC
Gallengangskarzinom	CCa
Gemischte konnektive Gewebserkrankung	MCTD
Gesunde Blutspender	HS
Gewebe spezifische Transglutaminase	tTG
Gliadin	Glia
Glomeruläre Basalmembran	GBM
Glycoprotein 2	GP2
Goodpasture Syndrom	GPS
Granulomatose mit Polyangiitis	GPA
Humane Epithelzelllinie-2	HEp-2
Immundiffusion	ID
Immunglobuline	Ig
Immunoblot	IB
Indirekte Immunfluoreszenz	IIF
Infektionsseren	INF
Konfidenz Intervall	CI
Labor-Informations-System	LIS
Lebertransplantation	LTx
Linien-Immunotest (line immuno assay)	LIA
Lupus erythematodes Zelle	LE Zelle

Mikropartikelbasierter Test	MIA
Mikroskopische Polyangiitis	MPA
Morbus Crohn	CD
Myeloperoxidase	MPO
Nationale Committee for Clinical Laboratory Standards	NCCLS
Primär sklerosierende Cholangitis	PSC
Proteinase 3	PR3
Radioimmunoassay	RIA
Radioimmunpräzipitation	RIP
Rasch progressive Glomerulonephritis	RPGN
Referenzbeads	SLR
Receiver Operating Characteristics	ROC
Ribonukleäres-Protein	RNP
Sjögren-Syndrom	SjS
Sjögren-Syndrom Antigene A/B	SSA/SSB
Smith Antigen	Sm
Systemischer Lupus erytemathodes	SLE
Systemisch autoimmun rheumatische Erkrankungen	SARE
Topoisomerase I	Scl-70
United States Center for Disease Control and Prevention	CDC
Variationskoeffizient	VK
World Health Organization	WHO
Zystische Fibrose	CF

Abbildungsverzeichnis

1 Einleitung

1.1 Autoimmunerkrankungen

1.1.1 Definition

Für den Begriff der „Autoimmunerkrankung“ stehen die Wörter „*auto*“ (griechisch) und „*immunis*“ (Latein) im Kontext, was im übersetzten Sinne so viel wie „eigen“ und „unempfindlich“ bedeutet. Im gesamten Wortzusammenhang erschließt sich eine Erkrankung mit einer Empfindlichkeit gegen Eigenes. Autoimmunerkrankungen (AIE) sind Dysregulationen des Immunsystems, gekennzeichnet durch eine Abwehrreaktion gegen körpereigene Bestandteile. Dabei werden diese entweder in ihrer Funktion beeinträchtigt oder gar degradiert [1]. Die Ausprägung einer AIE kann mehrere Ursachen haben, die einzeln oder im Zusammenhang wirken. Es können genetische Faktoren mit Defekten im immunregulatorischen Bereich sein oder äußere Faktoren, wie Virusinfektionen, Toxine oder Medikamente, die eine Dysfunktion auslösen. Mitunter können auch molekulare Mimikries zu einer Fehlinterpretation von vermeintlichen Fremdstrukturen führen [2].

Autoimmune Prozesse finden sowohl auf humoraler als auch auf zellulärer Ebene statt. Feltcamp beschrieb diese 1999 als eine „Erkrankung mit signifikant erhöhter Frequenz von Autoantikörpern in signifikant erhöhten Titern im Vergleich zu gesunden, regionalen, alters- und geschlechtsgemachten Kontrollen“ [3]. Diesen ergänzend finden sich zelluläre autoimmune Mechanismen in der Pathogenese wie z.B. T-lymphozytäre oder autoinflammatorische Komponenten, die bisher jedoch nicht für die Routinediagnostik relevant sind [4, 5, 6].

AIE können je nach Organbefall zwei erkrankungstypische Muster aufzeigen, welche sich über den Krankheitsverlauf in organspezifische und systemisch klinische Symptome manifestieren [7].

Die Prävalenz von AIE zeigt eine wachsende Tendenz. Derzeit sind circa 5-8% aller Individuen von einer AIE betroffen, jedoch steigt die Anzahl dieser Erkrankungen vor allem in den Industrieländern [8, 9]. Dieser Fakt lässt sich auf verschiedene artifizielle Umwelteinflüsse zurückführen sowie auf die Zunahme des durchschnittlichen Alters der Bevölkerung. Die wachsende Bedeutung dieser Erkrankungsform, die nach den Herz-Kreislauf und Tumorerkrankungen zu den dritthäufigsten chronischen Gesundheitsbeeinträchtigungen zählt, spiegelt sich in einer intensiven Grundlagenforschung auf diesem Gebiet wider. Die Entdeckung neuer Biomarker und die fortwährende Entwicklung diagnostischer Testverfahren stehen dabei im Vordergrund. Die Früherkennung und entsprechende Therapie dieser klinischen Entitäten können das Voranschreiten der autoimmunen Prozesse und deren schwerwiegende Manifestation vermeiden. Dabei ist das Auftreten von Antikörpern gegen körpereigene Strukturen, sogenannte Autoantikörper (AAk),

ein wesentliches Merkmal für das Vorhandensein einer AIE. Sie sind für die serologische Diagnostik von AIE essentiell.

1.1.2 Antikörper und ihre physiologische Rolle

Antikörper, auch als Immunglobuline (Ig) bezeichnet, sind Proteine, welche in B-Lymphozyten und deren ausdifferenzierter Form, in Plasmazellen, produziert werden. Sie bestehen aus zwei leichten und zwei schweren Ketten, die über Disulfidbrücken miteinander verbunden sind. Antikörper lassen sich in fünf Hauptklassen unterteilen, IgA, IgM, IgG, IgE und IgD (Abbildung 1). Diese Differenzierung bildet funktionelle Unterschiede im Immunsystem ab. Die Hauptaufgabe dieser Antikörper jeder Klasse ist es, spezifische Antigene mit Präzision zu binden und dadurch Effektormechanismen des Immunsystems auszulösen. Die gebundenen Antigene können durch die Antikörperbindung direkt neutralisiert oder durch Phagozytose der entstandenen Immunkomplexe beseitigt werden. Letzteres gibt dem Immunsystem ein Signal, dass körperfremde oder unzweckmäßige körpereigene Strukturen, sogenannte Antigene, anwesend sind, wodurch weitere angeborene und erworbene Immunantworten aktiviert werden. Antikörper können mit verschiedenartigen Strukturen wie zum Beispiel Proteine (Enzyme, Rezeptoren oder Strukturproteine), Desoxyribonukleinsäure (DNS), Phospholipide, Glykolipide oder Glykoproteine interagieren [10].

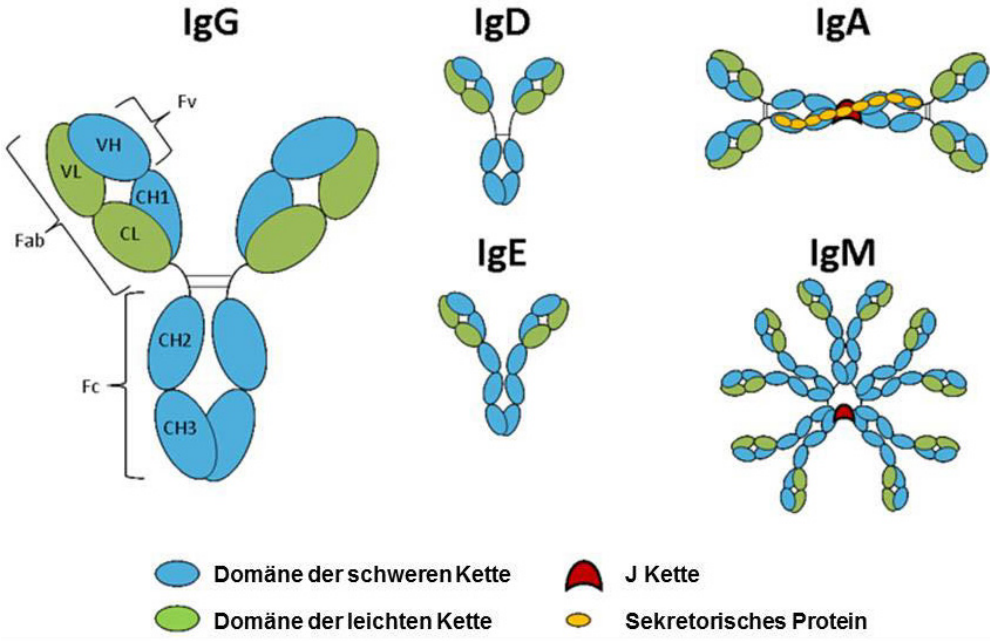

Abbildung 1: Schematische Darstellung des Aufbaus der Immunglobuline (Ig) A, G, M, E und D. IgG ist ein Monomer mit einer Bivalenz zu spezifischen antigenen Epitopen (MG = 146-170 kDa). Das Pentamer IgM besitzt zehn spezifische Paratope (MG = 900 kDa). Das IgA ist ein (sekretorisches) Dimer und kommt auf Schleimhäuten vor (MG = 385 kDa). IgD und IgE sind bivalente Monomere mit einem Molekulargewicht von 185 bzw. 190 kDa. Abbildung modifiziert nach [11].

Es gibt Wirbeltiere, wie Kamele und Haie, welche einen diversen Aufbau von Antikörpern aufzeigen [12, 13]. Diese besitzen neben den bekannten Hauptklassen auch *heavy chain-only* (englisch „nur schwere Kette besitzend“) Antikörper, so genannte Einzeldomänen-Antikörper. Diese natürlich vorkommenden, alternativen Antikörperformate weisen höhere Temperaturstabilität und höhere proteolytische Stabilität auf. Des Weiteren können diese schneller durch Gewebe diffundieren [14]. Diese genannten Eigenschaften machen die Einzeldomänen-Antikörper zum Einsatz für die Diagnosefindung in Testbestecken interessant [15].

1.1.3 Antikörper und ihre pathophysiologische Rolle

Antikörper können eine pathophysiologische Rolle ausüben, wenn sie gegen körpereigene, funktionelle Strukturen gerichtet sind. Diese nunmehr als AAk bezeichneten Ig, binden sogenannte Autoantigene. Manifestiert sich die Produktion von AAk mit einer pathogenetischen Funktion, kann sich eine AIE ausprägen, die ohne entsprechende Therapie zum Tode führt. Ohne vorläufige pathogenetische Wirkung zeigen AAk einen *Bystander-Effekt* (englisch „Zuschauer-Effekt“) und sind für die Diagnostik dennoch von Relevanz [16].

AAk spielen für die Diagnose von AIE eine entscheidende Rolle und sind auch für das Monitoring von AIE sehr wichtig. In einigen Fällen, wie zum Beispiel beim systemischen Lupus erythematodes (SLE), können spezifische AAk gegen doppelsträngige DNS (dsDNS) bereits vor dem Auftreten erster Symptome detektiert werden [17]. In diesem Zusammenhang können AAk auch eine prognostische Funktion aufweisen.

1.2 Diagnostik von Autoimmunerkrankungen

Die Diagnostik von AIE begann vor mehr als siebzig Jahren mit dem Auffinden der Lupus erythematodes (LE) Zelle in Patienten mit SLE [18]. Nachdem der Hintergrund der LE Zellentstehung geklärt war, gab es einen fortwährenden Aufwärtstrend in der Diagnostik von AIE.

1.2.1 Historische Entwicklung von Einzeltest-Formaten

Die Detektion von anti-nukleären Antikörpern (ANA) war bereits Mitte des vergangenen Jahrhunderts eine der ersten Methoden in der Diagnostik von AAk [18]. Dies revolutionierte die serologische Diagnose von systemischen AIE, im Speziellen den systemischen autoimmunen rheumatischen Erkrankungen (SARE) wie zum Beispiel den gemischten Bindegewebserkrankungen als auch SLE oder Sjögren-Syndrom (SjS). Die Pathogenese der SARE ist bis heute nicht vollkommen erschlossen und verstanden [19, 20]. Mit der Entdeckung und dem Einsatz von Fluoreszenzfarbstoffen in diagnostischen Testen konnten AAk mittels der indirekten Immunfluoreszenz (IIF) erstmals bestimmt werden [21]. Die IIF war die erste Technik, mit welcher ANA in Patienten mit SARE, speziell SLE, detektiert werden

konnten [22, 23, 24]. Als Substrat fungierten in erster Linie Gewebeschnitte der Rattenleber und später die humane Epithelzelllinie-2 (HEp-2), eine humane Larynxkarzinom-Zelllinie [10, 22, 24, 25, 26]. Für die Serologie von ANA stellen HEp-2 Zellen ein großes Spektrum an autoantigenen Zielstrukturen mit einer Vielzahl an klinisch relevanten Autoantigenen bereit [10, 27]. Bis heute stellt dieses Testformat den Goldstandard im Bereich der Diagnostik von SARE dar. Auch in anderen Bereichen, unter anderem den systemischen Vaskulitiden und den organspezifischen AIE, werden humane Zellen wie Granulozyten oder animalische Gewebeschnitte von Lunge, Niere oder Magen, bevorzugt aus Affe oder Ratte, als Goldstandard verwendet und in klinischen Laboratorien eingesetzt. Mit einem Fluoreszenzmikroskop können krankheitsspezifische Fluoreszenzmuster differenziert werden. Diese Muster geben einen Hinweis auf zugrundeliegende, klinisch relevante AAk-Spezifitäten [28]. Dieses Suchen von AAk-Spezifitäten nennt man auch Screening oder Suchtest.

Spezialisten können die entsprechende AAk-Spezifität anhand dieser Zellmuster gut abschätzen, jedoch erschweren Mischmuster oder überlappende Muster die Detektion von einzelnen ANA-Spezifitäten [10, 20]. Da differenzierte, diagnostische Aussagen entscheidend für eine adäquate Therapie der Patienten sind, muss mit einem Bestätigungstest die Spezifität von ANA genauer bestimmt werden. In Betrachtung der Historie zur Testentwicklung für SARE sind sich entwickelnde Parallelstrategien ersichtlich (Abbildung 2). Wie in Sowa *et al.* 2016 aufgeführt, kristallisierten sich verschiedene Methoden der ANA-Diagnostik heraus und wurden parallel zum Goldstandard IIF verwendet. Zum Beispiel konnten Smith (Sm)-AAk mit der Entwicklung der Immundiffusion (ID) neben den dsDNS-AAk als spezifischen Marker für SLE entdeckt werden. Jahre später wurden diese zu den diagnostischen Kriterien für die Bestätigung eines SLE hinzugefügt [29, 30, 31, 32, 33]. Jede der entwickelten Technologien hat ihre Vor- und Nachteile, wie in Tabelle 1 der Publikation Sowa *et al.* 2016 dargestellt. So entwickelten sich bis heute verschiedene Technologien, die das Ziel verfolgen so schnell und so präzise wie möglich, das klinisch-diagnostisch relevante Ergebnis zu erfassen.

Auch die Diagnostik von anti-neutrophilen zytoplasmatischen Antikörpern (ANCA) verlief in ihrer Historie ähnlich wie die der ANA. Definiert durch einen Konsensus von 1999 [34], ist der Goldstandard der ANCA-Serologie die IIF mit humanen Granulozyten, gefolgt von Bestätigungstesten mit extrahierten oder rekombinanten Antigenen wie der Enzyme-linked Immunosorbent Assay (ELISA), Immunoblot (IB) oder mikropartikelbasierte Teste (MIA) [35, 36, 37, 38, 39]. Im Gegensatz zur ANA-Diagnostik gab es für die ANCA-Diagnostik im Jahr 2017 einen neuen Konsensusvorschlag [40]. Diese Empfehlungen stellen nun die Notwendigkeit des zellbasierten Screenings in Frage. ANCA mit einer Spezifität gegen Proteinase 3 (PR3) oder Myeloperoxidase (MPO) sind hoch assoziiert mit einer bestimmten

Form der Kleingefäß-Vaskulitis und pathognomonisch für die Granulomatose mit Polyangiitis (GPA) bzw. die Mikroskopische Polyangiitis (MPA). Infolgedessen werden vor allem neu entwickelte immunometrische Drittgenerations-Teste wie der hoch sensitive PR3-ELISA für die serologische Diagnostik der ANCA-assoziierten Vaskulitiden (AAV) anstelle der noch weit verbreitete IIF empfohlen.

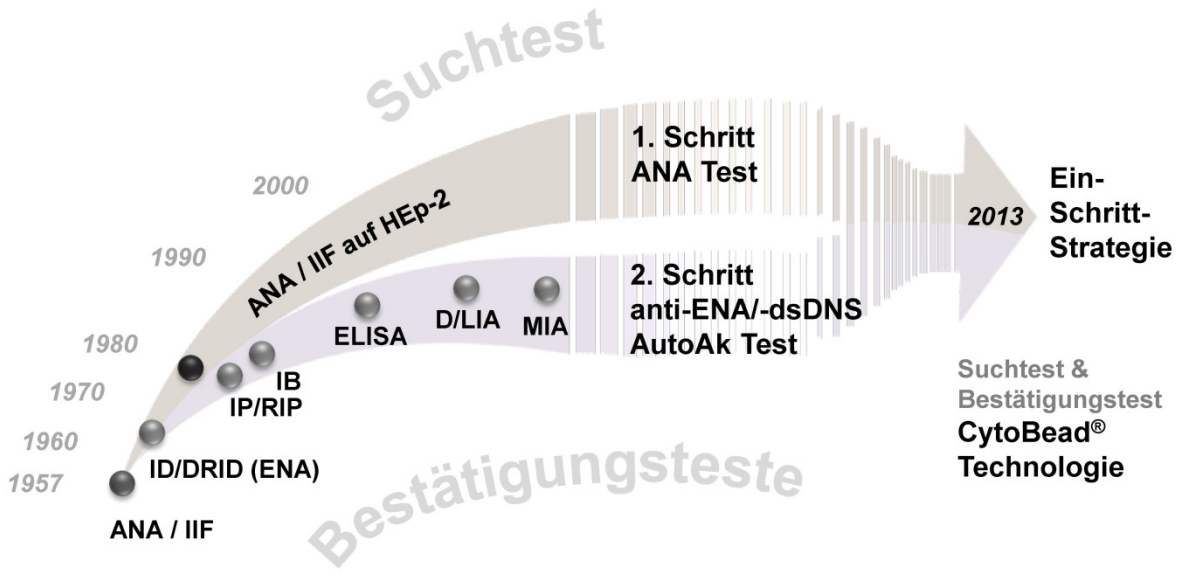

Abbildung 2: Schematische Darstellung der historischen Entwicklung von Autoantikörper (AAk)-Detektionsverfahren am Beispiel der Serologie von gemischten Bindegewebserkrankungen. Das Screening oder Suchen von AAk ist der erste Schritt in der Diagnostik. Zur Differenzierung der AAk-Spezifität wird ein Bestätigungstest nach dem Screening empfohlen. Diese Strategie wird als Zwei-Schritt-Strategie bezeichnet. Werden beide Verfahren vereinigt, spricht man von einer Ein-Schritt-Strategie. Eine der ersten kommerzialisierten Teste in diesem Kontext ist die CytoBead Technologie. Abbildung modifiziert nach [21].

ANA, anti-nukleäre Antikörper; *D/LIA*, Dot/LINE Immunoassays; *DRID*, Doppelt radiale Immundiffusion; *ELISA*, Ezyme-linked Immunosorbent Assay; *ENA*, extrahierbare nukleäre Antigene; *IB*, Immunoblot; *ID*, Immundiffusion; *IIF*, indirekte Immunfluoreszenz; *IP*, Immunpräzipitation; *MIA*, mikropartikelbasierter Test; *RIP*, Radioimmunpräzipitation

1.2.2 Profildiagnostik

Die Profildiagnostik ist eine effektive Methode, die biologische Heterogenität der AAk zu adressieren [20]. Das bedeutet, dass AAk im Organismus polyklonal existieren und sowohl gegen verschiedene Epitope eines Autoantigens als auch gegen mehrere autoimmune Zielstrukturen innerhalb einer autoimmunen Entität gerichtet sein können. Die Detektion dieser AAk-Reaktivitäten mit einem spezifischen Test ist eine Herausforderung und wird häufig nicht vollständig realisiert. Daher bedarf es mehreren serologischen Testungen, um das gesamte relevante AAk-Spektrum zu erfassen.

1.2.3 Autoantikörper-Teste mit nativen Substraten

Der Einsatz nativer Substrate zur Diagnostik von AAk, wie zum Beispiel Rattengewebeschnitte, stellte den Beginn der serologischen Autoimmundiagnostik dar und hatte bereits Mitte des zwanzigsten Jahrhunderts seinen Ursprung (Abbildung 2). Zum einen ist das große Spektrum von Epitopen zum anderen auch der Erhalt der nativen Epitop-Strukturen ein positiver Aspekt dieser Substrate. Dadurch können konformationsspezifische AAk als auch AAk gegen kontinuierliche Epitope detektiert werden. Solche Teste, wie der HEp-2 Zelltest zur Detektion von ANA, werden häufig als Screening Methode eingesetzt. Mit ihrer hohen diagnostischen Sensitivität (Quotient aus der Anzahl richtig positiver AAk Ergebnisse und der Gesamtheit der Patienten mit dieser AIE), jedoch geringeren diagnostischen Spezifität (Quotient aus der Anzahl richtig negativer Ergebnisse und der Gesamtheit der Probanden ohne diese AIE), ist die Wahrscheinlichkeit für das Auftreten eines falsch positiven Testergebnisses je nach Prävalenz der jeweiligen AIE vorhanden. Deshalb werden Teste mit gereinigten autoantigenen Strukturen bzw. sehr spezifische IIF-Teste wie der *Crithidia luciliae* Fluoreszenztest (CLIFT) für die dsDNS-AAk Analyse bevorzugt als Bestätigungstest eingesetzt.

1.2.4 Autoantikörper-Teste mit isolierten Autoantigenen

Extrahierte Antigene, ursprünglich aus nativen Substraten gereinigt oder rekombinant mittels Insektenzelllinien oder *Escherichia coli* hergestellt, werden für Bestätigungsteste eingesetzt. In den letzten 30 Jahren wurden dazu verschiedene Techniken entwickelt. Dabei haben sich unter anderem IB, ELISA, Radioimmunpräzipitation (RIP) oder MIA in der serologischen Diagnostik etabliert (Abbildung 2). Diese Techniken sind durch verschiedene Immunkomplextrennverfahren sowie Immobilisierungsstrategien (adsorptiv oder kovalent) gekennzeichnet. Für die serologische AAk-Diagnostik war die Entwicklung dieser Bestätigungsteste ein Zugewinn, denn nun konnten einzelne, klinisch relevante AAk-Spezifitäten detektiert und analysiert werden.

Für ein umfängliches Screening, bei welchem eine deutlich höhere Wahrscheinlichkeit auftritt, dass Individuen mit ähnlichen krankheitsrelevanten Symptomen im Rahmen der Differentialdiagnose von AIE getestet werden, ist es aus ökonomischer Sicht wichtig, einen schnellen, einfachen und kostengünstigen Test zu verwenden. Im Fall der Serologie der SARE wird hierzu der ANA-Screeningtest mit HEp-2 Zellen als autoantigenes Substrat verwendet. Im Anschluss müssen die positiven Ergebnisse mit mindestens einem Bestätigungstest kontrolliert werden. Die Entscheidung, welcher Test dafür verwendet wird, obliegt ausschließlich den Laborspezialisten, welche aufgrund der erkannten Fluoreszenzmuster einen Vorbefund angeben. Wird zum Beispiel ein homogenes Fluoreszenzmuster des Zellkerns der HEp-2 Zelle im Mikroskop ermittelt, muss die

Fluoreszenz der Mitose in der Metaphase den ausschlaggebenden Hinweis auf die mögliche AAk-Spezifität geben. Eine homogene Kernfluoreszenz und eine homogene Metaphasenfluoreszenz deuten auf dsDNS-AAk hin und damit auf einen möglichen SLE. Im Anschluss muss diese Vermutung durch die spezifische Bestimmung von dsDNS-AAk mit einem CLIFT, ELISA, RIP oder MIA bestätigt werden. Weiterhin korreliert das Auftreten der dsDNS-AAk mit dem Krankheitsverlauf der SLE, sodass eine kalibrierte Quantifizierung für ein Monitoring der Krankheitsaktivität bzw. des Therapieverlaufs essentiell ist [10]. Beide Testverfahren des Screenings und der Bestätigung ergänzen sich zu einer gezielten serologischen Diagnostik von AIE. Wenn beide im Rahmen der beschriebenen Stufendiagnostik eingesetzt werden, ist eine hohe Präzision der Ergebnisfindung garantiert [10].

1.2.5 Standardisierung von AAk-Bestimmungen

Eine wichtige Rolle in der serologischen Routinediagnostik von AIE spielt die Standardisierung von diagnostischen Verfahren und damit verbundene Ergebnisfindung. Für die Generierung von korrekten Testergebnissen basierend auf der Möglichkeit der Reproduktion innerhalb eines Labors bzw. zwischen unterschiedlichen Laboren mit unterschiedlichen Testkitherstellern sind internationale Referenzseren von hohem Interesse. Diese können in verschiedenen Formen in den Laboratorien integriert werden und tragen zur standardisierten serologischen Analyse von AIE bei. Allerdings existieren nur für wenige diagnostische Marker internationale Referenzseren. Bereits 1967 wurden erste Standards für die Laboratoriumsdiagnostik durch das Nationale Committee for Clinical Laboratory Standards (NCCLS) vorgeschlagen [41]. In den 70iger Jahren wurde das erste Referenzserum durch die World Health Organization (WHO) für die standardisierte Diagnostik von anti-Thyreoglobulin-AAk bereitgestellt. In den letzten vierzig Jahren folgten weitere Organisationen wie die United States Center for Disease Control and Prevention (CDC), welche weitere Referenzseren zur Verfügung stellten. Eine kalibrierte, quantitative Analyse von AAk ist mit diesen Referenzseren möglich. Da es sich bei Referenzseren um humane Seren handelt, sind diese limitiert verfügbar und müssen in Zukunft durch geeignete Alternativen ersetzt werden [20, 41]. Hersteller von Diagnostika etablieren deshalb hauseigene Referenzseren, die an internationalen Referenzseren abgeglichen wurden. Weiterhin müssen Hersteller von Testbestecken ihre hauseigenen Autoantigene, die als Substrat im Test eingesetzt werden, stets auf ein Referenzserum abgleichen und für hohe Reproduzierbarkeit sorgen. Dazu ist die Entwicklung von standardisierten Präparationstechniken der Autoantigene essentiell, vor allem wenn diese in ihrer Struktur sehr groß und komplex sind. Eine internationale Richtlinie für die Herstellung rekombinanter oder extrahierbarer Antigene existiert derzeit jedoch nicht. In Zukunft wäre die Erstellung eines solchen Konzeptes für eine Standardisierung von Testbestecken essentiell.

Weiterhin tragen automatisierte Messsysteme zur Abarbeitung und Evaluierung von diagnostischen Testbestecken zu einem kontinuierlichen, standardisierten Prozess im Labor bei. Angefangen bei der korrekten Zuordnung von Serumprobe zu Patient bis hin zur Ergebnisfindung und Dokumentation im Labor-Informations-System (LIS). Für die Bindegewebserkrankungen spielt die automatische Auswertung von IIF-Messergebnissen sowie IIF-Mustern eine wesentliche Rolle. Die hohe Vielfalt autoantigener Strukturen in HEp-2 Zellen oder Gewebeschnitten ist als sehr komplex einzuschätzen und bedarf eines weiten Erfahrungsspektrums. Automatisierte Messsysteme sind in der Lage objektive Ergebnisauswertungen vorzunehmen und unterstützen damit den subjektiven Menschen, der durch verschiedene innere und äußerliche Faktoren beeinflusst werden kann. Seit 2009, mit Einführung des AKLIDES® Systems (Medipan GmbH; Dahlewitz/Berlin), wurde die automatische, quantitative Auswertung von Fluoreszenzmustern kommerzialisiert [28, 32, 42, 43]. Diese Plattform zur digitalen Bildverarbeitung, welche auf der VideoScan Technologie (BTU Cottbus-Senftenberg; Senftenberg) aufbaut, verwendet mathematische Erkennungsalgorithmen, um spezifische Fluoreszenzmuster zu erkennen und objektive Testergebnisse zu generieren [44, 45, 46]. Beginnend mit der Ausgabe von klinisch relevanten ANA-Mustern (Abbildung 3) kann das AKLIDES® System im IIF-Bereich ebenfalls für die Mustererkennung von klinisch relevanten ANCA und dsDNS-AAk auf *Crithidia* spec., für die Ergebniserfassung von MIA und der automatischen Bildaufnahme von Gewebeschnitten eingesetzt werden [42, 43, 47, 48]. Die mathematischen Algorithmen generieren ein dreidimensionales Bild der zu analysierenden Objekte. Dabei werden Hintergrundsignale und Artefakte ausgeschlossen und Fluoreszenzintensitäten der zu analysierenden Objekte aufgenommen und konvertiert. Aufgezeichnete Fluoreszenzsignale werden mittels neu entwickelten Mustererkennungsalgorithmen zu dreidimensionalen Modellen verschiedener Basismuster umgerechnet (homogen, nukleolär, gesprenkelt, zentromer, Punkte, zytoplasmatisch; Abbildung 3). Diese dienen als Grundlage für die Befunderstellung.

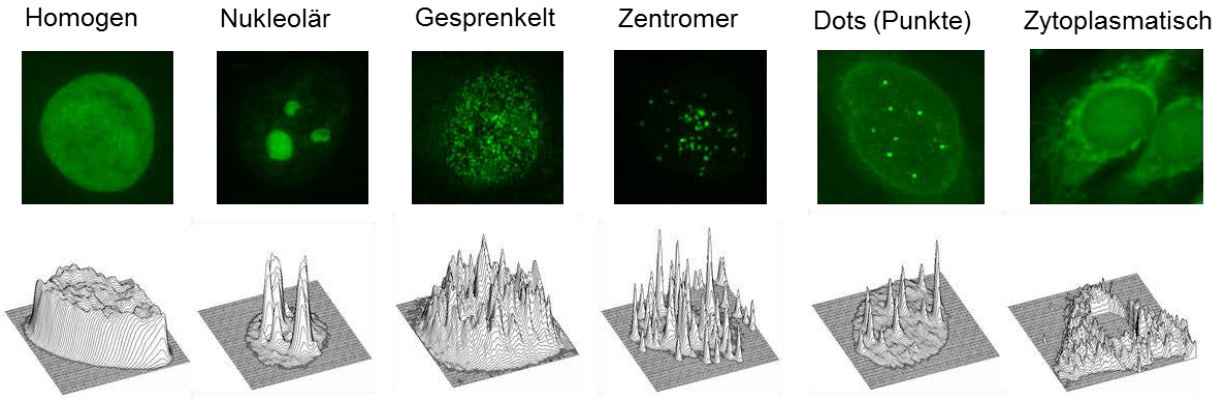

Abbildung 3: Schematische Darstellung der digitalen, fluoreszenzbasierten Bildverarbeitung des AKLIDES® Systems am Beispiel von krankheitsrelevanten ANA-Mustern. Mathematische Erkennungsalgorithmen generieren dreidimensionale Bilder und schließen Artefakte und Hintergrundsignale aus. Die errechneten dreidimensionalen Bilder verschiedener Muster (homogen,

nukleolär, gesprenkelt, zentromer, Punkte, zytoplasmatisch) dienen als Grundlage für die Befunderstellung in der Routinediagnostik von AIE. Abbildung modifiziert nach [28].

1.2.6 Entwicklung von Multiparametertesten

In den vorherigen Abschnitten wurde deutlich, dass Einzelteste nur durch eine ausgewählte Kombination diagnostische Sicherheit erlangen. Diagnostische Sicherheit ist maßgeblich für eine korrekte Diagnosestellung und damit für einen effizienten Therapieansatz.

Die bislang etablierte AAk-Stufendiagnostik in zwei Schritten ist ein probates Mittel und eignet sich zum Screening mit anschließenden Bestätigungstesten. Diese sukzessive Abhandlung diagnostischer Schritte ist aber auch eine zeit- und kostenaufwendige Prozedur. Häufig werden dazu mehrere Einzelantigen-Teste wie z.B. ELISA zur Diagnosefindung benötigt und erhöhen damit den Zeit- und Materialeinsatz. Multiplexteste wie der MIA oder Linien-Immunotest (LIA) sind durch ihre simultane Analyse von mehreren AAk-Spezifitäten in einem Testansatz im Routinelabor weit verbreitet. Sie bieten einen ökonomischen Vorteil und werden häufig als Bestätigungstest in der Zweistufendiagnostik eingesetzt.

Eine simultane Analyse mehrerer Parameter bzw. verschiedener Analysesysteme in einem Testansatz ist ein Multiparametertest. Als eine weitere Ebene der serologischen Diagnostik von AIE können relevante Teste der Zweistufendiagnostik in eine Einstufendiagnostik überführt werden. Durch die Entwicklung neuer Technologien, wie zum Beispiel der in dieser Dissertation dargelegten CytoBead Technologie, wurde eine neue Generation von Multiparametesten etabliert (Abbildung 2). Diese Neuentwicklung garantiert eine sichere, kostengünstige und zeitsparende Diagnosefindung und trägt in Einzelfällen zur Vermeidung von falsch interpretierten Screening-Ergebnissen bei [21]. Neben den ökonomischen Vorteilen ergeben sich weitere Vorteile im pädiatrischen Bereich, der wenig Ausgangsmaterial zur Verfügung stellen kann. Demzufolge können komplette AAk-Serologien wie die von SARE und anderen AIE gezielt durch multiparametrische Profildiagnostik klinisch relevanter AAk-Kombinationen umgesetzt werden. Diese garantieren eine verlässliche (Früh-)Diagnostik, Prognostik und Theranostik, um eine individualisierte, medizinische Patientenversorgung und Therapie zu ermöglichen (Abbildung 4) [49].

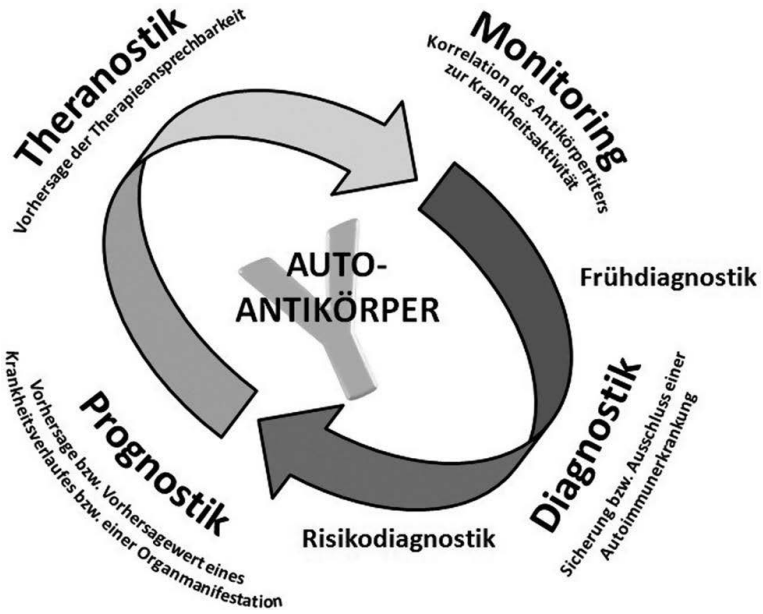

Abbildung 4: Schematische Darstellung der klinischen Relevanz von Autoantikörpern. Erkrankungsspezifische Autoantikörper sind richtungweisend in der (Früh)Diagnostik der assoziierten Autoimmunerkrankung. Darüber hinaus können sie wertvolle Hinweise bezüglich Erkrankungsentwicklung, Therapieansprechen und Krankheitsaktivität liefern [49].

Eine in Tabelle 1 der Publikation Sowa *et.al* 2016 veröffentlichte Darstellung der bis dato vorhandenen Multiplex- und Multiparameterteste zeigt, wie unterschiedlich diese in Testmatrix und Messmethode aufgebaut sein können. Im Kapitel 1.2.2 wurde auf die Standardisierung von Testergebnissen auf Basis von international anerkannten Referenzseren eingegangen. Anhand dieser Referenzseren müssen Hersteller von Testbestecken nicht nur hausinterne Referenzseren und einzusetzende Autoantigen-Substrate abgleichen, sondern auch die Einstellung des Testformats ist entscheidend. Zusätzlich werden spezielle Analysesysteme benötigt, wie beispielsweise das AKLIDES® System, um eine objektive und quantitative Auswertung dieser Testformate zu gewährleisten [21, 28, 31, 47, 49, 50].

2 Zielstellung

Der steigende Bedarf an Autoimmundiagnostik (AID) erfordert die Entwicklung von effizienten Teststrategien unter Einschluss multiparametrischer Testverfahren. Diese neuen Methoden müssen durch klinische Studien evaluiert und ihre Standardisierung garantiert werden. Die Standardisierung der Diagnostik von AAk ist bislang noch ungenügend adressiert.

Die Zielstellung der vorliegenden Dissertation war die Entwicklung und Evaluierung einer multiparametrischen Technologie (i) für die serologische Analyse von systemischen Vaskulitiden und (ii) die Erweiterung dieser Methode als Notfalltest zur serologischen Diagnostik der rasch progressiven Glomerulonephritis (RPGN). Zur Evaluierung sollten international ausgelegte, klinische Studien durchgeführt werden. Beide Testsysteme sollten durch die Verwendung internationaler Standards und durch die automatische Analyse mit dem AKLIDES® System integrationsfähig im heutigen Diagnostikalltag sein. Dementsprechend mussten Leistungsdaten der Teste wie diagnostische Sensitivität und Spezifität sowie intra- und inter-Chargenschwankungen mit denen der Einzeltestformate kompatibel sein.

Eine weitere Aufgabenstellung der vorliegenden Dissertation gestaltete sich in der Entwicklung und Evaluierung eines Screeningtests zur Detektion von anti-Glykoprotein 2 (GP2)-AAk im Rahmen der serologischen Diagnostik von autoimmunen Lebererkrankungen. Diese Untersuchungen sollten als Vorarbeit für die Entwicklung eines weiteren Multiparametertests für organspezifische AIE durchgeführt werden. Dabei wurden mit GP2 transduzierte HEp-2 Zellen als Testplattform etabliert und evaluiert. Die Evaluierung sollte durch internationale, klinische Studien mit Patientenseren, schwerpunkthaft mit primär sklerosierender Cholangitis (PSC), zur Abklärung der möglichen Assoziation von GP2-AAk mit dem klinischen Phänotyp der PSC, durchgeführt werden.

3 Ergebnisse

Innovationen werden durch translationale Forschung gefördert. Die vorliegende kumulative Dissertation zeigt in insgesamt fünf Publikationen mit Erstautorenschaft (inklusive eine geteilte Erstautorenschaft) die Umsetzung von Innovationen im Rahmen der definierten Aufgabenstellung. Die Entwicklung und Evaluierung einer neuen Multiparametertechnologie auf der Basis der digitalen, fluoreszenzbasierten Bildverarbeitung, die in der Lage ist komplexe Aufgabenstellungen zu bewältigen, ist der Kern der vorliegenden Ergebnisse.
Ein weiterer Schwerpunkt der Arbeit betrifft die Erhöhung der diagnostischen Sicherheit zur Diagnose von AIE. Es wurden multiparametrische Testsysteme zur Diagnostik von AAV und der rasch progressiven Glomerulonephritis (RPGN) etabliert [31, 51]. Im Rahmen der Entwicklung wurden jeweils international ausgelegte, klinische Studien durchgeführt. In einer weiteren Veröffentlichung von Scholz *et al.* 2015 wurde ein Multiparametertest zur Diagnosefindung von Bindegewebserkrankungen evaluiert [50]. Folglich der gesamten Reihe an Multiparametertesten auf der Grundlage der CytoBead Technologie sind weitere Publikationen mit methodischer Ausrichtung veröffentlicht worden [21, 49].

3.1 Der CytoBead Test – eine Multiparametertechnolgie als diagnostisches Werkzeug nächster Generation für die Diagnostik von AIE

In der serologischen Diagnostik von AIE gibt es eine große Variabilität von AAk, sodass sich viele verschiedene AAk-Profile für unterschiedliche AIE manifestieren können. Die folgenden methodisch ausgelegten Publikationen Sowa *et al*. [21, 49] präsentieren die strategische Ausrichtung der Entwicklung dieser Multiparametertechnologie.

3.1.1 *Next-Generation Autoantibody Testing by Combination of Screening and Confirmation — the CytoBead Technology* (Sowa *et al*. 2016)

Im Bereich der serologischen Diagnostik von SARE, vor allem von Bindegewebserkrankungen, müssen umfangreiche AAk-Profile durch die verwendete Teststrategie abgebildet werden (Abbildung 2). Durch die kontinuierliche Entdeckung neuer AIE-spezifischer Markerproteine sind diese Herausforderungen noch gewachsen. Beginnend bei der Basis der AAk-Diagnostik, der IIF auf Gewebeschnitten bzw. HEp-2 Zellen, die als sehr sensitive Teste zum Screening eingesetzt wurden, folgten zur Bestimmung der AAk-Spezifität hoch spezifische Teste, sogenannte Bestätigungsteste. In Tabelle 1 der Publikation Sowa *et al*. 2016 sind die AAk-Detektionsmethoden in der Routinediagnostik von SARE zusammengetragen und jeweils deren Vor- und Nachteile sowie deren Testprinzip und Anwendungsbereiche angegeben. Über die letzten Jahrzehnte haben sich das Screening und dessen Bestätigung technisch immer mehr voneinander entfernt und erforderten das Vorhalten von unterschiedlichen Techniken in der Routinediagnostik. Beispielsweise, ein positiver HEp-2 Zelltest mit homogenem Fluoreszenzmuster des

Zellkerns wird im rheumatologischen Labor diagnostiziert und zur Bestimmung der AAk-Spezifität im Radioisotopenlabor durch einen Radioimmunoassay (RIA), dem immer noch derzeit anerkannten Goldstandard zur Bestimmung von dsDNS-AAk, verifiziert. Durch die Entwicklung der Multiparametertechnologie CytoBead konnten die Nachteile der Zweischrittdiagnostik überwunden und alle zur vollständigen Diagnostik benötigten Teste miteinander in einer Testumgebung kombiniert werden (siehe Abbildung 5). Wurde eine dsDNS-AAk positive Serumprobe aufgetragen, konnte in der Mitte der Auftragsstelle ein homogenes Fluoreszenzmuster der HEp-2 Zellen detektiert werden. In der Peripherie befinden sich vier Mikropartikelkompartimente, die jeweils zwei Mikropartikelpopulationen mit jeweiligen autoantigenen Zielstrukturen in sich tragen. Eine sichtbare Ringfluoreszenz der MIA konnte in diesem Beispiel in Kompartiment III auf der kleineren Mikropartikelpopulation (dsDNS) beobachtet werden. Alle Ergebnisse dieses Multiparametertests wurden allein durch eine einmalige Auftragung der Serumprobe ermittelt: im Screening ein homogenes Muster und in der Bestätigung ein dsDNS-AAk. Folglich könnte dieser serologische Befund an den behandelnden Rheumatologen übermittelt werden, der in Kombination mit weiteren krankheitsspezifischen Symptomen die Grundlage für eine sichere Diagnosestellung mit anschließender Therapieverordnung erhält.

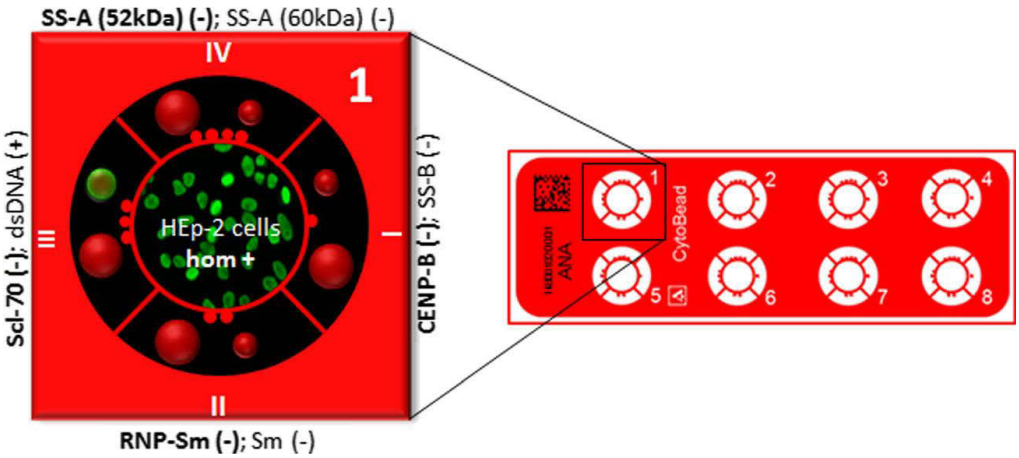

Abbildung 5: Schematische Darstellung des CytoBead ANA zur Bestimmung von AAk in SARE. Beispielsweise wurde hier eine dsDNS-AAk positive Serumprobe verwendet: Im Screeningtest mit HEp-2 Zellen (Mitte der Auftragstelle) konnte ein homogenes Zellkernmuster detektiert werden. Im Bestätigungstest (Peripherie der Auftragstelle, Kompartiment III) wurde eine positive Mikropartikelfluoreszenz, bewertet durch einen grün fluoreszierenden Ring der dsDNS-beschichteten Mikropartikel, detektiert. Alle weiteren Kompartimente (I, II, IV) zeigten keine Fluoreszenz.

Unter der Beachtung von Leitlinien und internationalen Empfehlungen im Zuge der CytoBead Entwicklungen, wie dem internationalen Konsensus zur ANCA Diagnostik von Savige *et al*. 1999 [34], konnte diese Technologie für einen adäquaten Einsatz in klinischen Routinelaboren optimiert werden. Eine lokale Trennung der Bearbeitungsbereiche einzelner Techniken zur Diagnosefindung war nun nicht mehr notwendig.

3.1.2 Der CytoBead-Assay – Eine neue Möglichkeit der multiparametrischen Autoantikörperanalytik bei systemischen Autoimmunerkrankungen (Sowa *et al*. 2014) / *The CytoBead assay – a novel approach of multiparametric autoantibody analysis in the diagnostics of systemic autoimmune diseases* (Sowa *et al*. 2015)

Anknüpfend an der zuvor dargestellten Übersichtspublikation (Sowa *et al.* 2016) werden in diesen methodisch ausgelegten Publikationen die krankheitsspezifischen CytoBead Teste dargestellt. Durch die Verwendung von Glasobjektträgern mit definiert designter Teflonmaske war es möglich, verschiedene Parameter miteinander zu kombinieren. Jede der acht einzelnen Auftragstellen des Objektträgers wurde in der Mitte mit dem Bereich für den Screeningtest und in der Peripherie mit den Bereichen für die Bestätigungsteste ausgestattet (siehe Abbildung 6). Die Zusammenstellung der jeweiligen Screening- und Bestätigungsteste erfolgte unter Betrachtung der aktuellen Leitlinien und Literaturbeiträge und dem bilateralen Austausch mit Laborärzten, die in den Publikationen als Co-Autoren aufgeführt wurden.

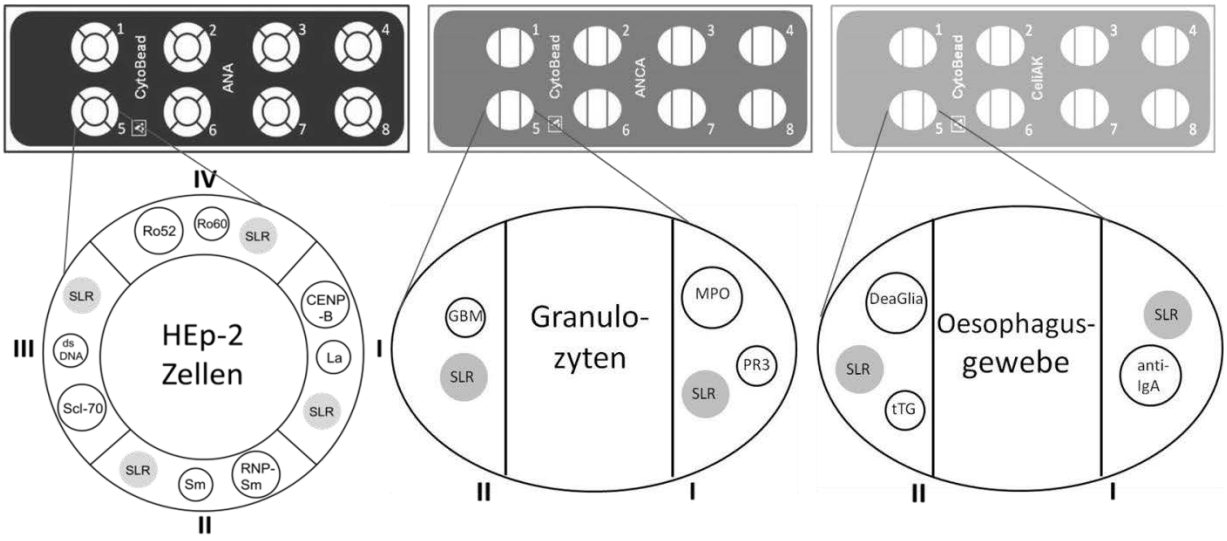

Abbildung 6: CytoBead Objektträgerausführungen mit acht Auftragsstellen für unterschiedliche Testprofile. Kombination aus Screeningtest mit nativem Substrat der Zellen oder Gewebeschnitt (Zentrumskompartiment) und antigenbeladene, fluoreszierende Mikropartikel (periphere Kompartimente). CytoBead ANA (links), ANCA (mittig) und Zöliakie (rechts). SLR ist die Bezeichnung der Referenzpartikel für die manuelle Mikropartikelklassifikation.

Der Screeningtest des CytoBead ANCA basierte klassisch auf Ethanol fixierte, humane Granulozyten, zu welchem ein neues Isolationsprotokoll mit Immobilisierung und Fixierung erstellt wurde. Eine 70% Ethanolfixierung zeigte adäquate Resultate zu kommerziellen Testen, die in der Publikation Sowa *et al*. 2014 dargestellt sind [31]. Die Screeningteste des CytoBead ANA und CytoBead CeliAK basieren auf anderen Zell- und Gewebesubstraten. Der CytoBead ANA verwendete HEp-2 Zellen, welche auf dem Glasobjektträger kultiviert und nach 24 Stunden mit einer Methanol / Aceton Behandlung für fünf Minuten fixiert wurden. Für das Screening von Zöliakie-spezifischen AAk mit Hilfe des CytoBead CeliAK

wurde Ösophagus-Gewebe aus Primaten verwendet und Kryoschnitte von 8 µm Dicke auf die Glasoberfläche gebracht. Beide Screeningteste zeigten adäquate Ergebnisse zu kommerziellen Testen, wie in den Publikationen von Scholz *et al*. 2015 und Grossmann *et al*. 2016 veröffentlicht [47, 50].

Die Bestätigungsteste der CytoBeads wurden durch Mikropartikel aus Polymethylmethacrylat (PMMA) entwickelt. Es wurden rot fluoreszierende (Red5 50) Mikropartikel von zwei sich in ihrer Größe (Durchmesser 9 µm und 15 µm) unterscheidenden Populationen verwendet. Die kovalente Kopplung der Antigene konnte durch 1-ethyl-3-(3-dimethylaminopropyl)carbodiimid-Hydrochlorid (EDC-HCl)-Aktivierung erreicht werden. Die Konzentration des EDC wurde auf 10 bis 100 mM eingestellt. Nach 15 minütiger Aktivierung der Carboxylgruppen auf der Mikropartikeloberfläche wurde eine bestimmte Kopplungsmenge der Antigene von 2 bis 10 µg pro 200.000 Beads hinzugegeben. Der Kopplungspuffer musste für jedes Antigen individuell optimiert werden. Im Allgemeinen erfolgte die Kopplung über drei Stunden bei Raumtemperatur. Für die Bestätigung spezifischer AAk wurden für den CytoBead ANCA als antigene Zielstrukturen PR3, MPO und später auch das Glomeruläre Basalmembran (GBM)-Antigen gekoppelt. Für den CytoBead ANA waren dies Sjögren-Syndrom Antigene (SSB, SSA), Centromer-B Protein (CENP-B), Smith Antigen (Sm), Ribonukleäres-Protein (RNP), dsDNS, Topoisomerase I (Scl-70) und für den CytoBead CeliAK die Gewebetransglutaminase (tTG), deamidiertes Gliadin (DeaGlia) und anti-IgA. Der Vergleich dieser MIA mit kommerzialisierten Testen zeigte gute bis sehr gute Übereinstimmungen, welche in den vorher angeführten Publikationen dargestellt wurden [31, 47, 50].

Auswertung der CytoBead Teste

Die CytoBead Teste wurden entwickelt um zwei verschiedene Auswertungsmethoden der IIF zu gewährleisten. Die Verwendung des grün fluoreszierenden Farbstoffs Fluoresceinisothiocyanat (FITC) für die Antikörperkonjugate und die Verwendung von zwei verschieden großen, rot fluoreszierenden Mikropartikelpopulationen ermöglichte sowohl die Auswertung im klassischen Fluoreszenzmikroskop per Auge als auch automatisch unter Verwendung der digitalen, fluoreszenzbasierten Bildverarbeitung im AKLIDES® System.

Für die klassische Auswertung per Auge wurden unterstützend Referenzpartikel implementiert. Diese Mikropartikel sind grün fluoreszierend und 12 µm im Durchmesser. Wurde eine Probe als negativ bewertet und zeigte keine Fluoreszenzsignal auf der antigenbeschichteten Mikropartikeloberfläche, konnte die Lokalisation des Mikropartikel-bereichs trotzdem durch die Präsenz der immergrünen, fluoreszierenden Referenzpartikel detektiert werden. Wurde eine Probe als positiv bewertet und waren grün fluoreszierende Ringe der antigenbeschichteten Mikropartikel sichtbar, konnte mithilfe der Referenzpartikel entschieden werden, welche der beiden Population ein positives Signal zeigte.

Für die automatische Auswertung wurden jeweils vier Kalibratoren für jedes Antigen und jeden CytoBead Test etabliert. Diese Kalibratoren wurden aus hausinternen Standards gewonnen, welche an internationale Standards, z.B. denen vom CDC zur Detektion von anti-PR3 AAk, anti-MPO AAk sowie anti-tTG AAk und der World Health Organization (WHO) zur Detektion von anti-dsDNS AAk, angepasst wurden. Mittels nichtlinearer Regression und der asymmetrischen Fünf-Parameter- bzw. Richards Funktion wurden Masterkurven generiert [55]. Diese Masterkurven konnten zur Umrechnung der im AKLIDES® System ermittelten willkürlichen Einheiten [AU] in quantitativ kalibrierte U/ml bzw. IU/ml verwendet werden. Wie in Abbildung 7 dargestellt, wurden die Kalibratoren Cal0 bis Cal3 für spezielle Abschnitte der sigmoidal verlaufenden Masterkurve ausgewählt. Sie sind so ausgewählt worden, dass sie den Wendepunkt zum Anstieg, die exponentielle Phase, die lineare Phase und den Wendepunkt in die Plateauphase abdeckten. Ein spezielles Softwaretool des AKLIDES® Systems machte es möglich die Masterkurve für jeden Testdurchlauf entsprechend der ermittelten Fluoreszenzintensität der Kalibratoren prozentual anzupassen. Geräte- und Chargenschwankungen konnten durch dieses Verfahren ausgeglichen werden.

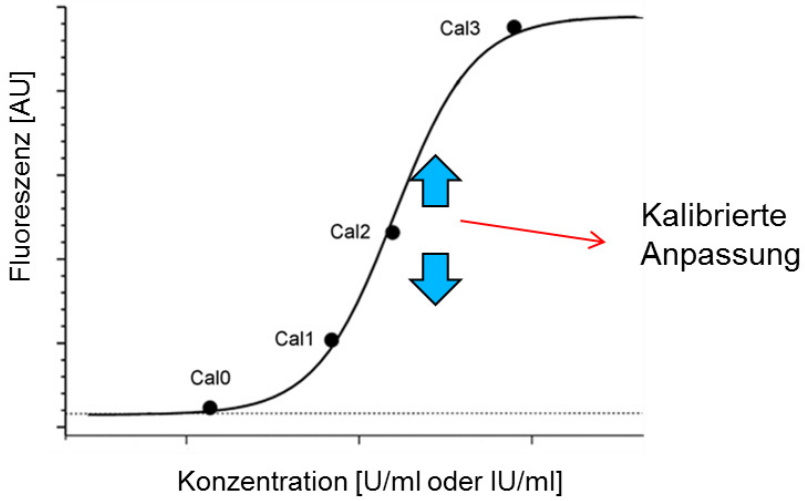

Abbildung 7: Schematische Übersicht einer Titrationskurve eines Kalibrationsserums mit definierten Kalibratorpositionen (Cal0 bis Cal3). Die vier Kalibratoren sind in den Bereichen des Wendepunktes zum Anstieg, der exponentiellen Phase, der linearen Phase und des Wendepunktes der Plateauphase festgelegt. Sie dienen zum Ausgleich von Geräte- und Chargenschwankungen und werden automatisch durch ein entsprechendes AKLIDES®-Softwaretool durch Verwendung von Masterkurven angepasst.

3.1.3 *Simultaneous Automated Screening and Confirmatory Testing for Vasculitis-Specific ANCA* (Sowa *et al*. 2014)

Das Konzept der CytoBead Technologieentwicklung wurde zuerst durch die Entwicklung des CytoBead ANCA umgesetzt. Wie bereits in Kapitel 3.1.2 dargestellt, ist der CytoBead ANCA ein Multiparametertest bestehend aus einer Kombination von Screening und Bestätigung. Entsprechend einem neu entwickelten Isolationsverfahren von Granulozyten aus frischem, humanen Blut und einer Fixierungsstrategie mit 70% Ethanol wurde gleichzeitig die

Kompatibilität für die Reaktionsbedingungen der Antigenbeschichtung auf Mikropartikeln realisiert.

Goldstandard des Screenings ist die Ethanolfixierung von Granulozyten

Durch die 70% Ethanolfixierung wurden die Zellmembranen der Granulozyten und die Membranen derer Granula perforiert. Dies führte zur Wanderung der intrazellulären Proteine entsprechend ionischer Wechselwirkung. Kationische Proteine wie MPO migrierten unter diesen Bedingungen in Richtung Kernmembran zur negativ-geladenen DNS. Als Ergebnis zeigten MPO-AAk eine perinukleäre Fluoreszenz in der IIF. Neutral-geladene Proteine wie PR3 verblieben im Zytoplasma, was zu einer zytoplasmatischen Fluoreszenz in der IIF führte (siehe Abbildung 8).

Mikropartikel als feste Phase für das Bestätigungssystem

Die Herstellung von antigenbeschichteten Mikropartikeln aus PMMA wurde durch EDC-Aktivierung von Carboxylgruppen auf der Mikropartikeloberfläche und der anschließenden Peptidbindung der Proteine PR3 bzw. MPO realisiert. Während der IIF-Reaktion reagierten anti-PR3 bzw. anti-MPO spezifische AAk aus humanem Serum mit den antigenbeschichteten Mikropartikeln. Die Zugabe eines anti-human IgG spezifischen Sekundärantikörpers, welcher mit FITC konjugiert war, führte zur Anfärbung der gebundenen AAk in Form einer ringförmig, grünen Fluoreszenz an der Mikropartikeloberfläche. In Abbildung 8 sind die beiden häufigsten klinischen Fälle im Bereich der AAV dargestellt. Durch Zugabe von Serumproben mit anti-PR3 (links) und anti-MPO AAk (rechts) wurde im Screeningtest eine zytoplasmatische (links) und perinukleäre (rechts) Fluoreszenz der Granulozyten detektiert. Im Bestätigungstest konnten jeweils anti-PR3 (links) und anti-MPO (rechts) AAk durch eine positive Ringfluoreszenz der Mikropartikel detektiert werden. Folglich konnte die gesamte Diagnostik der AAV durch eine einmalige Auftragung der Serumprobe durchgeführt werden.

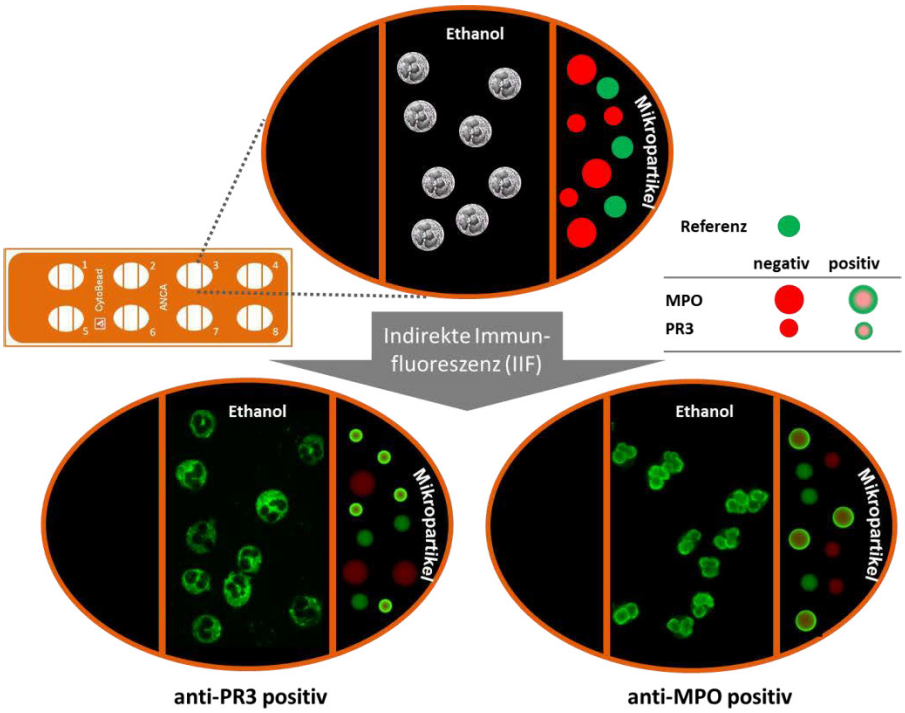

Abbildung 8: Schematische Darstellung des Prinzips des CytoBead ANCA. Ein speziell designter Glasobjektträger mit acht Auftragstellen zu je drei Kompartimenten wurde für die Umsetzung angefertigt. In der Mitte der Auftragstelle befinden sich humane Granulozyten, welche mit 70% Ethanol fixiert wurden (Goldstandard). Die in der Peripherie (rechts von der Mitte) mit PR3 (9 µm Partikel) und MPO (15 µm Partikel) beschichteten Mikropartikel zeigen eine positive Fluoreszenz für anti-PR3 (links; Granulozyten mit zytoplasmatischem Muster und 9 µm Mikropartikel mit grünem Fluoreszenzring) und anti-MPO (rechts; mit perinukleärem Muster und 15 µm Mikropartikel mit grünem Fluoreszenzring).

Die Umsetzung dieser Multiparameterdiagnostik wurde durch eine Anpassung von Isolation und Fixierung, einer neuen Immobilisierungsstrategie von Granulozyten und Mikropartikel, der Optimierung von Antigenbeschichtungsmenge und Umgebungsbedingungen, der Angleichung von Serumverdünnung (1:20 Vorgabe laut Leitlinie [34]) und Konjugatverdünnung sowie neu designten Objektträgern mit Teflonmaske realisiert. Die Feinjustierung von diagnostischer bzw. relativer Sensitivität und Spezifität erfolgte durch Zugabe von Zusätzen wie Rinderserum-Albumin oder Tween-20 in den jeweiligen Verdünnungspuffern der Serumproben und des Konjugats.

Klinische Studie und Testparameter

Im Rahmen einer klinischen Studie wurden insgesamt 592 humane Seren getestet, welche 118 Patienten mit AAV, 125 gesunde Blutspender (HS) und 349 Kontrollpatientenseren umfassten (siehe Tabelle 1 Sowa *et al*. 2014).

Mittels Receiver Operating Characteristics (ROC) Kurvenanalyse wurde eine Grenzwert- bzw. Cut-Off-Bestimmung durchgeführt. Damit wurde der Test zur optimalen Diskriminierung zwischen positiven und negativen Befunden eingestellt (siehe Abbildung 9). Zur ROC-Analyse wurden Patienten mit PSC und Colitis Ulcerosa (UC) aus der gesamten Kohorte

aufgrund ihrer bereits bekannten Präsenz von vor allem PR3-AAk ausgeschlossen. Die ROC-Kurvenanalyse ergab für anti-PR3 AAk einen Cut-Off von 0,9 IU/ml (Fläche unter der Kurve [AUC] 0,896) und für anti-MPO AAk 3,0 IU/ml (AUC 0,934) bei einer diagnostischen Spezifität von 95%.

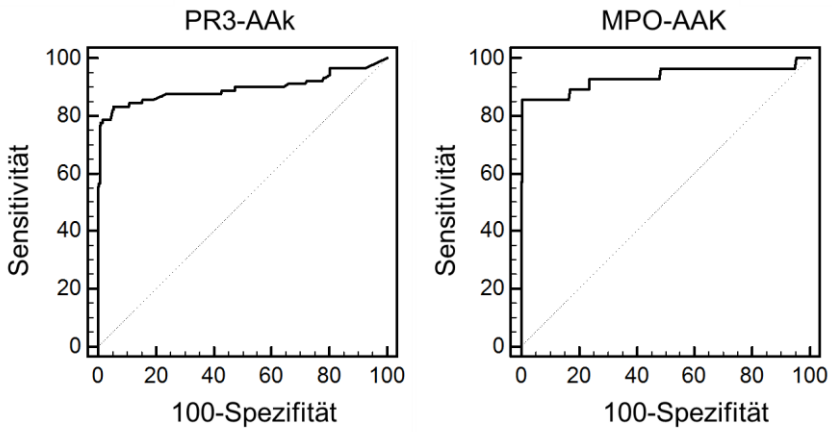

Abbildung 9 Receiver Operating Characteristics (ROC) Kurvenanalyse von PR3- und MPO-AAk im CytoBead ANCA. Als positives Kriterium für PR3-AAk wurden 90 Patienten mit GPA und für MPO-AAk 28 Patienten mit MPA verwendet. Als negatives Kriterium wurden 125 HS, 133 Rheumatoide Arthritis, 49 Infektionserkrankungen, 20 Morbus Crohn und 20 Autoimmune Hepatitis Patienten in die Analyse eingeschlossen.

Es wurden kommerzielle Teste wie Ethanol fixierte bzw. Formalin fixierte Granulozyten und anti-PR3 bzw. anti-MPO AAK ELISA mit dem CytoBead ANCA verglichen. Der Vergleich der kommerziellen Teste zum CytoBead ANCA zeigte gute bis sehr gute Übereinstimmungen (siehe Tabelle 2 und 3 sowie Abbildung 3 und 4 Sowa *et al.* 2014). Im Detail konnte eine sehr gute Übereinstimmung des klassischen Screeningtests mit dem CytoBead ANCA mittels statistischer Interrater-Reliabilitäts-Analyse (Cohens Kappa-Wert) ermittelt werden (k = 0,876 95% Konfidenzintervall [CI]: 0,812 – 0,940 für das perinukleäre Fluoreszenzmuster und k = 0,820 95% CI: 0,755 – 0,844 für das zytoplasmatische Fluoreszenzmuster). Im Bestätigungstest gab es gute Übereinstimmungen durch Cohens Kappa-Wert-Ermittlung für anti-PR3 AAk (k = 0,775 95% CI: 0,710 – 0,839) und für anti-MPO AAk (k = 0,720 95% CI: 0,7596 – 0.843). Wurde der Gesamtansatz des CytoBead ANCA mit der Mehrstufendiagnostik der klassischen Teste verglichen, konnte eine sehr gute Übereinstimmung ermittelt werden (k = 0,831 95% CI: 0,777 – 0,885).

Weiterhin zeigte die Studie die Reproduzierbarkeit des CytoBead ANCA innerhalb einer Charge (intra-Assay Variationskoeffizient [VK]) und zwischen mehreren Chargen (inter-Assay VK) mit < 15%. Die funktionelle Test-Sensitivität wurde für anti-PR3 AAk bei 0,6 IU/ml und für anti-MPO AAk bei 2,5 IU/ml ermittelt und lag damit unter dem ermittelten Cut-Off der Testparameter.

Ein zusätzlicher Aspekt innerhalb dieser Studie war der Vergleich des manuellen und automatisch ausgewerteten Endpunkt-Titers per Auge bzw. mit dem AKLIDES® System. Manuell wurde der Endpunkt-Titer durch sequenzielle Titration auf entsprechende Serumverdünnungen von 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, 1:5120 und durch Verwendung eines manuellen Fluoreszenzmikroskops der Firma Motic (Volksrepublik China) ermittelt. Automatisch wurde der Endpunkt-Titer in einer Serumverdünnung von 1:20 mittels AKLIDES® System und entsprechender AKLIDES® CytoBead ANCA Software errechnet. Es konnte gezeigt werden, dass beide Varianten der Endpunkt-Titer-Ermittlung eine sehr gute Übereinstimmung aufwiesen (siehe Tabelle 4 Sowa *et al*. 2014). Der ermittelte Cohens Kappa-Wert lag bei 0,985 (95% CI: 0,980 – 0,991).

3.1.4 *Simultaneous comprehensive multiplex autoantibody analysis for rapidly progressive glomerulonephritis* (Sowa *et al*. 2016)

Der CytoBead RPGN wurde auf Basis des CytoBead ANCA entwickelt. Die klinische Assoziation von ANCA mit dem Goodpasture Syndrom (GPS) bzw. von anti-dsDNS AAK mit dem SLE stellte die Grundlage für diese Weiterentwicklung dar. Dieser Multiparameter-Test umfasst die Kombination der benötigten fünf Parameter für die Durchführung einer vollwertigen, serologischen Diagnostik der RPGN (IIF mit Ethanol fixierten Granulozyten in der Mitte der Auftragstelle sowie MIAs mit PR3, MPO, GBM und dsDNS beladenen Mikropartikeln in der Peripherie der Auftragstelle; siehe Abbildung 1 Sowa *et al.* 2016). Die quantitative Analyse der Ergebnisse mittels AKLIDES® System konnte durch den Einsatz von Kalibratoren aus Standards umgesetzt werden. Für PR3, MPO und dsDNS konnten internationale Standards verwendet werden. Für GBM wurde ein hausinterner Standard, welcher mit IIF auf Nierengewebe, LIA und ELISA abgeglichen wurde, eingesetzt. Somit konnte die quantitative Analyse der anti-PR3, anti-MPO und anti-dsDNS AAk in IU/ml und der GBM-AAk in U/ml erreicht werden.

Klinische Studie und Testparameter

Innerhalb der klinischen Studie wurden insgesamt 287 humane Seren untersucht. Davon waren 90 von Patienten mit AAV, 42 mit SLE, 43 mit GPS sowie 57 Kontrollpatienten und 55 HS zu analysieren. Zur Ermittlung der spezifischen Cut-Offs unter der Voraussetzung einer 95% diagnostischen Spezifität der einzelnen Parameter wurde eine ROC-Kurvenanalyse durchgeführt (siehe Abbildung 3 Sowa *et al*. 2016). Als Cut-offs wurden 5 IU/ml für jeweils anti-PR3 AAk und anti-MPO AAk ermittelt, sowie 7 U/ml für anti-GBM AAk und 10 IU/ml für anti-dsDNS AAk. In dieser Konstellation wurde ein intra-Assay VK und ein inter-Assay VK von < 15% ermittelt (siehe Tabelle 2 Sowa *et al*. 2016). Für die VK-Analyse der IIF konnte ein Wert < 23% erhoben werden.

Die Prävalenz der AAk gegen PR3, MPO, GBM und dsDNS in den verschiedenen Kohorten der klinischen Studie wurde in Tabelle 3 der Publikation aufgeführt. Weiterhin ist ein Box-

Whisker-Plot zur grafischen Darstellung dieser Prävalenzen und zur Darstellung der Mediane einzelner Kohorten angefertigt worden (siehe Abbildung 10). Zusätzlich wurde im Kruskal-Wallis Test bestätigt ($p < 0,005$), dass der CytoBead RPGN signifikant unterschiedliche Werte in den Patienten- und Kontrollgruppen zeigte. Im CytoBead RPGN wurde eine Prävalenz von 85,0% für anti-PR3 AAk in Patienten mit GPA, 77,1% für anti-MPO AAk in Patienten mit MPA, 88,4% für anti-GBM AAk in Patienten mit GPS und 83,3% für anti-dsDNS AAk in Patienten mit SLE detektiert. In Kontrollgruppen wie zum Beispiel in HS und Infektionsseren (INF) konnten Prävalenzen nur zwischen 0,0% bis 5,4% und 0,0% bis 3,5% erhoben werden.

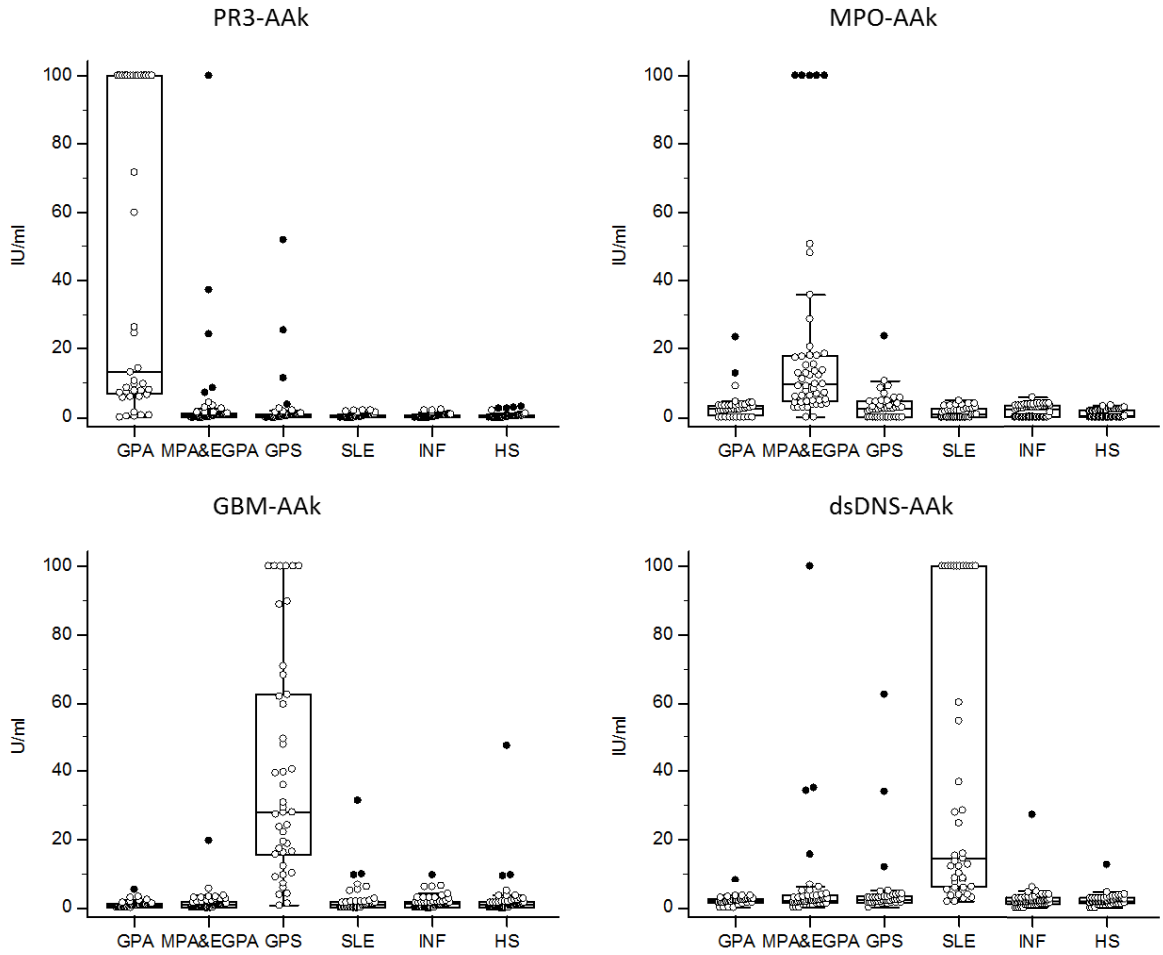

Abbildung 10: Darstellung der detektierten AAK gegen PR3, MPO, GBM und dsDNS der Bestätigungsteste mittels MIA im Box-Whisker-Plot. Testung von 287 humanen Seren von 90 AAV Patienten (GPA, MPA und Eosinophiler Granulomatose mit Polyangiitis [ESGPA], 42 mit SLE, 43 mit GPS sowie von 57 Infektionsseren (INF) und 55 Seren von HS.

Die Interrater-Reliabilitäts-Analyse (Cohens Kappa-Wert) ergab eine sehr gute Übereinstimmung von anti-PR3 AAk ($k = 0,852$ 95% CI: 0,762 – 0,941), anti- MPO AAk ($k = 0,803$ 95% CI: 0,710 – 0,896) und anti-GBM AAk ($k = 0,824$ 95% CI: 0,731 – 0,917) mit klassischen ELISA Testen.

Der anti-dsDNS AAk MIA zeigte eine moderate Übereinstimmung zum klassischen ELISA (k = 0,500 95% CI: 0,387 – 0,612). Hierbei wurden 52/287 (18,1%) diskrepante Ergebnisse ermittelt (McNemar Test: Differenz 12,54%; 95% CI: 7,94 – 15,62; p < 0,0001). Der Vergleich der diagnostischen Sensitivität und Spezifität zeigte eine bessere Korrelation des MIA (diagnostische Sensitivität 83,3% / Spezifität 97,3%, siehe Tabelle 6 Sowa *et al.* 2016) zum Krankheitsbild als der klassische ELISA (diagnostische Sensitivität 85,7% / Spezifität 84,8%).

3.2 Entwicklung eines Screeningtests zur Detektion von GP2-AAk als neuen, sensitiven Marker in Patienten mit PSC

Als Vorarbeit für eine weitere CytoBead Entwicklung im Rahmen der serologischen Diagnostik der PSC wurde ein Screeningtest auf Basis GP2 transduzierter HEp-2 Zellen entwickelt und evaluiert. In der anschließenden klinischen Studie wurde die Assoziation der Präsenz von anti-GP2 AAk in Patienten mit PSC und dem klinischen Phänotyp untersucht.

3.2.1 *Mucosal autoimmunity to cell-bound GP2 isoforms is a sensitive marker in PSC and associated with the clinical phenotype* (Sowa *et al*. 2018)

Transduktion

Für den Aufbau des Screeningtests wurde eine Transduktion von HEp-2 Zellen mit GP2 durchgeführt. Hierfür wurde das Lentivirus-System verwendet. Jede transduzierte Zelle trug jeweils eine der bislang identifizierten vier humanen GP2 Isoformen, fortlaufend bezeichnet als $GP2_{1\text{-}4}$ [52, 53, 54]. Eine weitere transduzierte Zelle trug einen Leervektor, um eine Kontrollzelle zu generieren. Durch spezifische anti-$GP2_{1\text{-}4}$ Antikörperreaktionen auf der Membranoberfläche der transduzierten Zellen konnte die Expression und Membranbindung des $GP2_{1\text{-}4}$ in der Durchflusszytometrie geprüft werden (siehe Abbildung 1 Sowa *et al.* 2018, Durchführer der Transduktion: Rafal Kolenda).

Aufbau der IIF

Anders als der HEp-2 Screeningtest im CytoBead ANA wurden die transduzierten HEp-2 Zellen für 48 Stunden auf dem Glasobjektträger kultiviert und anschließend in 4% Paraformaldehyd-Fixierungslösung für 60 Minuten behandelt. Im Nachgang wurden $GP2_{1\text{-}4}$-AAk hausinterner Referenzseren aufgetragen, um die Präsenz der membrangebundenen GP2-Isoformen zu prüfen (siehe Abbildung 11). Nach einer Inkubationszeit von 60 Minuten wurde mit einem anti-human IgA spezifischen, FITC-gekoppelten Konjugat für weitere 60 Minuten inkubiert. Mittels AKLIDES® System oder einem manuellen Fluoreszenzmikroskop der Firma Motic wurden die Fluoreszenzmuster bewertet. Ein Ergebnis wurde als positiv befundet, wenn ein membranständiges Fluoreszenzmuster beobachtet werden konnte. Im Gegensatz dazu wurde ein Ergebnis als negativ bewertet, wenn intra- oder extrazelluläre, zytoplasmatische bzw. mitotische Fluoreszenzsignale detektiert wurden. So zeigte Patient 1

eine starke, spezifische Membranfluoreszenz mit der transduzierten Zelle $GP2_1$ und eine schwache, spezifische Membranfluoreszenz mit der transduzierten Zelle $GP2_2$. Patient 2 zeigte eine starke, spezifische Membranfluoreszenz mit der transduzierten Zelle $GP2_4$ und eine schwache, spezifische Membranfluoreszenz mit der transduzierten Zelle $GP2_3$. Der gesunde Blutspender hatte kein spezifisches Fluoreszenzmuster. Alle hausinternen Standards präsentierten kein Fluoreszenzmuster auf der Kontrollzelle.

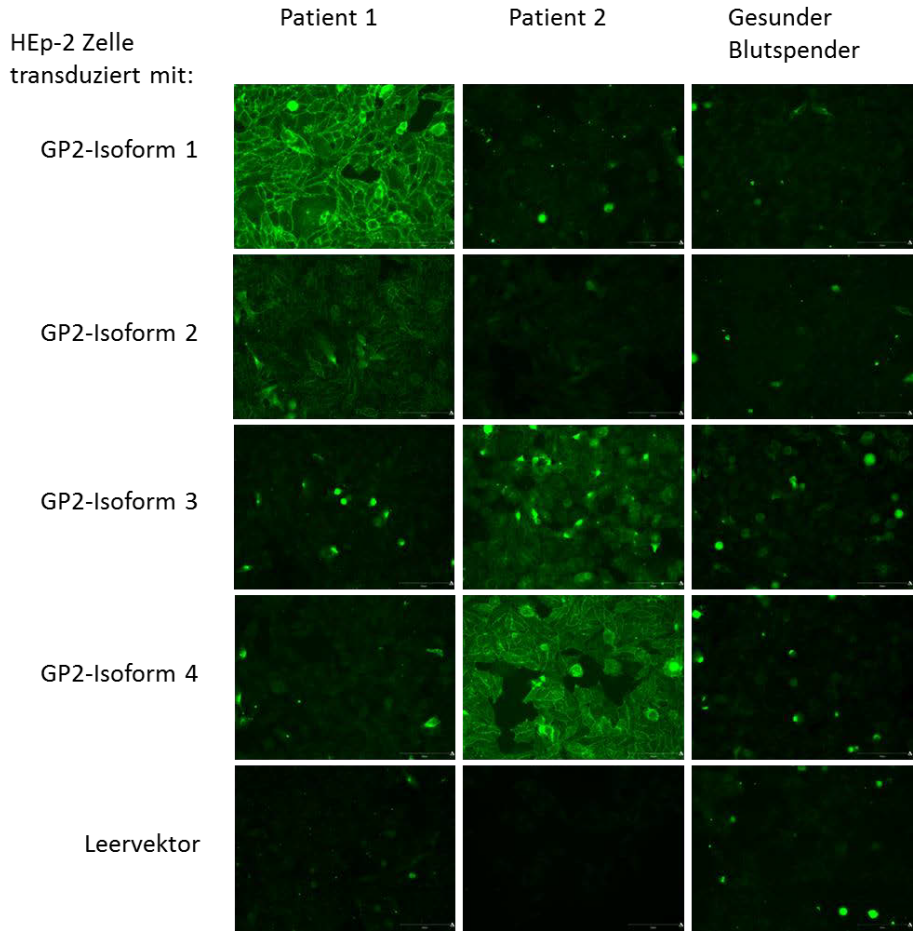

Abbildung 11: IIF zur Detektion von anti-$GP2_{1-4}$ IgA. Exemplarisch wurden zwei Patientenseren und ein gesunder Blutspender dargestellt, die als hausinterne Standardseren zur Prüfung der Präsenz der membrangebundenen GP2-Isoformen eingesetzt wurden. Patient 1 zeigte eine starke, spezifische Membranfluoreszenz mit der transduzierten Zelle $GP2_1$ und eine schwache, spezifische Membranfluoreszenz mit der transduzierten Zelle $GP2_2$. Patient 2 zeigte eine starke, spezifische Membranfluoreszenz mit der transduzierten Zelle $GP2_4$ und eine schwache, spezifische Membranfluoreszenz mit der transduzierten Zelle $GP2_3$. Der gesunde Blutspender wies kein spezifisches Fluoreszenzmuster auf. Alle Seren präsentierten kein Fluoreszenzmuster auf der Kontrollzelle.

Klinische Studie

Die klinische Studie wurde mit 212 PSC Patienten und 95 Kontrollpatienten mit Zystischer Fibrose (CF) sowie 50 HS durchgeführt (siehe Tabelle 1 der Publikation Sowa *et al*. 2018). Für alle Seren wurden der Nachweis von IgG und IgA spezifischen AAk durchgeführt (siehe Tabelle 2 Sowa *et al.* 2018). Generell zeigten IgA gegen die jeweiligen GP2 $Isoformen_{1-4}$ eine signifikant höhere Spezifität als IgG. Die höchste Diskriminierungsrate von PSC Patienten

und getesteten Kontrollgruppen konnte durch die Nachweise von anti-$GP2_1$ IgA (47,2%) und anti-$GP2_4$ IgA (48,6%) erzielt werden. Wenn beide AAk-Nachweise miteinander kombiniert wurden, konnte eine signifikant höhere Sensitivität (66,0%) im Vergleich zu den Einzelanalysen erzielt werden ($p_1 < 0{,}0001$ und $p_4 = 0{,}0004$). Dies kennzeichnete gleichzeitig auch die höchste Prävalenz für den Nachweis von anti-GP2 IgA und weiterhin den besten Youden-Index (YI) von 0,64 als Testparameter.

Weiterführend wurde die Korrelation der Präsenz der anti-$GP2_{1\text{-}4}$ IgG und IgA mit einem begleitenden, klinischen Phänotyp der PSC ermittelt. Durch den Exakten Test von Fisher wurden Patienten mit Zirrhose, Gallengangskarzinom (CCa), Lebertransplantation (LTx), Autoimmunhepatitis (AIH) und entzündlichen Darmerkrankungen (IBD) wie UC und Morbus Crohn (CD) untersucht (siehe Tabelle 3 Sowa *et al.* 2018). Zusätzlich wurden mittels logistischer Regression unabhängige Variablen wie anti-$GP_{1\text{-}4}$ IgG und IgA sowie Alter und Geschlecht zur Abschätzung des Risikos für das Auftreten bei einer Zirrhose, CCa und LTx bewertet (siehe Tabelle 4 Sowa *et al.* 2018). Für die abhängige Variable „Zirrhose“ konnten unabhängige Variablen wie „anti-$GP2_{1+4}$ IgA“ sowie „Alter“ und „männliches Geschlecht“ als Risikofaktoren statistisch belegt werden. Im Gegensatz dazu konnte für die abhängige Variable „CCa“ die unabhängige Variable „anti-$GP2_3$ IgG“ als Risikofaktor ermittelt werden. Für die abhängige Variable „LTx“ hingegen zeigten die unabhängigen Variablen „Zirrhose“ und „UC“ eine positive Assoziation und die unabhängigen Variablen „anti-$GP2_2$ IgA“ und „AIH“ eine negative Assoziation.

4 Diskussion

In den letzten Jahrzehnten entwickelten und etablierten sich verschiedene Methoden zur serologischen Diagnostik von AIE, welche unter der Beachtung bestimmter Vorschriften zur Anwendung kommen [19, 34]. Dabei etablierte sich die Zweistufendiagnostik von AIE, definiert durch eine sukzessive Durchführung von Screening und Bestätigung(en). Diese Art der Diagnostik verursacht einen hohen Zeit- und Kostenaufwand und veranlasst in einigen Fällen eine räumliche Trennung der Testdurchführung. Neue Ansätze aus dem Bereich der Effizienzsteigerung sind Multiplexteste, welche für den Einsatz als Bestätigungstest geeignet sind, jedoch eine Zweistufendiagnostik nicht vereinfachen können. Eine Einstufendiagnostik als Multiparametertest mit einer Kombination aus Screening und Bestätigung(en) ist eine maximale Effizienzsteigerung. Jedoch ist dieses Verfahren für die Anwendung in der Routinediagnostik von AIE mit ihren hohen Standardisierungsanforderungen nicht realisiert.

Im Rahmen der vorliegenden Dissertation sollte ein Multiparametertest entwickelt werden, welcher wesentliche Vorteile im Zeit- und Kostenmanagement sichern kann. Weiterhin sollten internationale Leitlinien, Referenzseren und eine automatische Auswertung zur Standardisierung von Ergebnissen für die Routinediagnostik von AIE integriert werden. Zusätzlich sollte durch die automatisierbare Auswertung eine Hochdurchsatzanalyse realisierbar sein, um einen Einsatz in jedem Routinelabor zu gewährleisten.

4.1 Der CytoBead – ein Multiparametertest als diagnostisches Werkzeug nächster Generation für die Diagnostik von AIE (Sowa *et al*. 2014 & 2015 & 2016)

Eine sich zukünftig in der klinischen Routinediagnostik von AIE etablierende Innovation ist die CytoBead Technologie. Sie definiert eine Kombination aus Screening und Bestätigung(en) und dient zur effektiven Umsetzung von klinische Leitlinien [19, 34]. Das Testformat ist die IIF, die aufgrund der hervorragenden Sensitivität zur serologischen Diagnostik von AIE eingesetzt wird. Im CytoBead Screeningtest werden native Testsysteme wie Zellen und Gewebestrukturen auf speziell designten Glasobjektträgern fixiert. Die Bestätigungsteste, die ebenfalls auf den Glasobjektträgern immobilisiert werden, bestehen aus artifiziellen Testsystemen, der MIAs. In der Herstellung können verschiedene Reaktionsbedingungen für jede Zielstruktur optimal genutzt werden, bevor sie für das Gesamtkonstrukt zusammengefügt werden. Somit entsteht ein Multiparametertest mit optimalen Substratbedingungen. In dieser Kombination wird das Patientenmaterial nur einmalig aufgetragen und es werden mehrere Ergebnisse gleichzeitig erhalten. Anders als in der bisherigen Zweistufendiagnostik ist mittels CytoBead eine Einstufendiagnostik möglich.

Generell ist die Anzahl der zur Diagnosefindung eingesetzten Teste maßgebend zur Erhöhung der Sicherheit eines korrekten Befundes. Besonders der Einsatz von Bestätigungstesten grenzt das sich darstellende Krankheitsbild im Kontext der vorliegenden Differentialdiagnose ein. Doch ist es für nationale und internationale Routinelabore schwierig, alle verwendeten Teste und entsprechend zu prüfenden Parametern beim Kostenträger finanziell geltend zu machen. Eingesendete Materialien werden durch entsprechend diagnostische Untersuchungsaufträge der behandelnden Ärzte bearbeitet. Demzufolge ist eine aussagekräftige Diagnostik des Patientenmaterials aus Patientensicht und aus ökonomischer Sicht ein wichtiger Aspekt des Routinelabors. Ziel ist im Einklang aller beauftragten, diagnostischen Untersuchungen den geringsten temporären und logistischen Aufwand zu betreiben und ein korrektes Ergebnis mit hohem Reproduktionswert für das spätere Monitoring der Patienten ermitteln zu können. Für die Zertifizierung eines Diagnostiklabors durch den Gesetzgeber sollte vor allem letzteres nachweisbar sein. Die CytoBead Technologie verinnerlicht eine kalibrierte Ergebnisfindung durch internationale Referenzseren und trägt damit zur höchsten Präzision und internationalen Vergleichbarkeit bei.

Auswertestrategien der CytoBead Technologie

Oft sind neue Methoden zur Diagnosefindung nur durch Anschaffung von neuen Messsystemen in Laboratorien implementierbar. Doch hohe Investitionskosten und geringe Raumkapazität verhindern in einigen Fällen die Anschaffung neuer Technologien. Im Routinelabor für Autoimmunerkrankungen sind Fluoreszenzmikroskope für die fluoreszenzbasierten Mustererkennungen unabdingbar. Seit mehr als 70 Jahren wird in jedem dieser Labore die klassische, manuelle IIF durch FITC-Signaldetektion durchgeführt. Demzufolge wurden die Testkomponenten der CytoBead Technologie strategisch ausgewählt, um sowohl manuelle als auch automatische Auswertbarkeit zu garantieren. Die Mikropartikel tragen eine Rotfärbung, die manuell nicht sichtbar ist, aber für die automatische Auswertung zur Fokussierung und Sortierung der Mikropartikel essentiell ist. Die DAPI-Zellkernfärbung als Bestandteil des Eindeckmediums ist für native Substrate zur automatischen Fokussierung und Definition von Zellkernen im AKLIDES® System erforderlich. Der Sekundärantikörper ist mit einem FITC-Fluoreszenzmolekül konjugiert und demzufolge für die manuelle und automatische Auswertung von Fluoreszenzmustern geeignet. Demnach können sowohl semi-quantitative Ergebnisse durch manuelle Auswertung als auch quantitative Ergebnisse durch automatisierte Auswertung mittels AKLIDES® System erzielt werden.

Die quantitative Auswertung des CytoBead auf der Basis der digitalen, fluoreszenzbasierten Bildverarbeitung mittels AKLIDES® System wird durch eine zusätzliche Kalibrierung zu einem international vergleichbaren Test. Mittels Fünf-Parameteranalyse werden definierte,

chargenspezifische Masterkurven ermittelt, welche als Barcode auf dem Analysezertifikat des Testbestecks (Kit) beigelegt sind [55]. Diese werden bei Erstanwendung der jeweiligen Charge im AKLIDES® System eingescannt. Durch Auslesen der Messwerte der vier Kalibratoren jedes CytoBead Tests wie zum Beispiel für den CytoBead RPGN Kalibrator 1 bis 4 (anti-PR3, anti-MPO-, anti-dsDNS und anti-GBM) als Mischserum, sind die Masterkurven prozentual ausgleichbar. Dazu müssen die Kalibratoren in jedem Testlauf mitgeführt werden. Mit dieser Masterkurvenanpassung können temporäre Fehler in der Abarbeitung oder verschiedene räumliche Bedingungen (Temperatur, Luftfeuchtigkeit) egalisiert werden.

CytoBead ANCA (Sowa et al. 2014)

Seit mehr als 25 Jahren werden ANCA zur serologischen Diagnostik von AAV verwendet. Die IIF mittels Ethanol fixierter, neutrophiler Granulozyten ist seither der Goldstandard für das Screening von ANCA. Bei positivem Screening Ergebnis werden Bestätigungsteste wie die Formalin fixierten Granulozyten in der IIF oder Festphasenteste wie ELISA bzw. MIA zur Bestimmung der AAk-Reaktivität eingesetzt. Im Unterschied zu Ethanol fixierten Granulozyten zeigen Formalin fixierte Granulozyten keine perinukleären oder nukleären Muster, da das Vernetzungsreagenz Formalin keine Perforation der Membranstrukturen verursacht. Formalin fixierte Granulozyten sind daher als Bestätigungstest oder sekundärer Screeningtest im Routinealltag der Diagnostik von AAV zu finden.

Ein wesentlicher Vorteil der Festphasenteste, welche mit extrahierten oder rekombinanten Antigenen beladen sind, ist die quantitative Analyse der AAk. Demnach werden quantitative Ergebnisse nach dem Screening erhoben. PR3- und MPO-AAk sind pathognomonisch für GPA und MPA und korrelieren mit dem Krankheitsverlauf und deren Aktivität. Für das Monitoring der Patienten im Therapieverlauf ist der quantitative Nachweis dieser AAk sehr relevant. Demzufolge wird ein hohes Maß an Präzision und Reproduzierbarkeit an den einzusetzenden diagnostischen Test gestellt, um eine sehr gute Vergleichbarkeit der Ergebnisse über mehrjährige Therapiezeitläufe zu sichern.

Im Rahmen der klinischen Studie wurden kommerzielle Teste wie Ethanol fixierte bzw. Formalin fixierte Granulozyten und anti-PR3 bzw. anti-MPO ELISA mit dem neu entwickelten CytoBead ANCA verglichen. Der Vergleich zeigte gute bis sehr gute Übereinstimmungen und sehr gute Reproduzierbarkeiten (< 15%) innerhalb einer Charge und zwischen mehreren Chargen. Im Hinblick auf Testgeschwindigkeit und Materialeinsatz ist der CytoBead ANCA deutlich effizienter als kommerzielle Teste. Alle Ergebnisse wurden aus einem Testansatz erhoben und nicht aus vier Einzeltesten. Des Weiteren war es möglich atypische ANCA (aANCA) in Patienten mit IBD zu ermitteln. Sie zeigten in der IIF ein atypisches Muster und konnten von den anti-PR3 und anti-MPO MIA nicht als AAV der GPA oder MPA bestätigt werden. Unter Verwendung kommerzieller Teste hätte es nach IIF zusätzlich einer ANCA-

Bestimmung durch Formalin fixierte Granulozyten oder / und der anti-PR3 ELISA und anti-MPO ELISA bedurft, die für die Ergebnisfindung jedoch wesentlich mehr Zeit und Material in Anspruch genommen hätten. Weiterhin konnte in der Studie gezeigt werden, dass der anti-PR3 MIA sensitiver als der anti-PR3 ELISA war. Patienten mit UC und PSC zeigten PR3-AAk, die mittels ELISA nicht detektiert werden konnten, jedoch in dieser Patientengruppe zu finden sind [56, 57].
Eine weitere Untersuchung zeigte eine sehr gute Übereinstimmung der manuellen Endpunkt-Titeranalyse durch den Menschen und der automatischen Endpunkt-Titeranalyse durch das automatisierte AKLIDES® System. Demnach kann der Einsatz des CytoBead ANCA in Zusammenarbeit mit der automatisierten Auswertung in Hochdurchsatz-Kliniken mit einer hohen Zahl an Patienten mit Verdacht auf AAV realisiert werden.

CytoBead RPGN (Sowa et al. 2016)

Die RPGN ist namentlich und klinisch gesehen eine rasch auftretende und aggressiv verlaufende AIE, die selektiv oder in kombinierter Form von Lunge und Nieren auftreten kann. Binnen Stunden bzw. weniger Tage kann diese zum Verlust der Nierenfunktion oder zum Tode führen, insofern der Patient nicht schnellstmöglich korrekt diagnostiziert und therapiert wird. Allein klinische Symptome reichen zur Differentialdiagnose nicht aus. Zur korrekten, klinischen Indikation des gesamten Krankheitsspektrums von drei auftretenden Krankheitstypen bedarf es einer serologischen Diagnostik. Typ I der RPGN wird als anti-GBM Erkrankung bezeichnet, in welcher anti-GBM AAk nachzuweisen sind. Typ II ist eine Immunkomplex-vermittelte Erkrankung, welcher eine AIE wie z.B. SLE zugrunde liegt. Typ III wird als Pauci-Immun-Vasculitis bezeichnet. Dieser Typus, der mehr als 50% aller RPGN-Fälle abdeckt, ist durch das Auftreten von AAK gegen PR3 und MPO definiert [58]. In ca. 10-30% dieser Patienten ist eine Kombination von AAK gegen GBM, PR3 und MPO zu finden, was als Indikator für eine progressive Form der Erkrankung steht. Eine Einstufendiagnostik, umgesetzt im CytoBead RPGN, ist für die serologische Diagnostik der RPGN sehr vorteilhaft. Eine schnelle Ergebnisfindung, einhergehend mit einem schnelleren Therapiestart, sichert dem Patienten höhere Überlebenschancen.
In der klinischen Studie konnte sowohl eine sehr gute Vergleichbarkeit zu klassischen Testen wie IIF und ELISA gezeigt werden als auch eine gute bis sehr gute Übereinstimmungen zum Krankheitsbild. Einzig die Vergleichbarkeit der Testformate ELISA und MIA zur Analyse von dsDNS-AAk zeigte nur moderate Übereinstimmung. Beim Vergleich der diagnostischen Sensitivität und Spezifität wurde jedoch deutlich, dass der anti-dsDNS MIA im CytoBead RPGN die bessere Korrelation zum Krankheitsbild aufwies als der anti-dsDNS spezifische, klassische ELISA. Ein wesentlicher Unterschied beider Testmethoden war die Bindung der dsDNS auf der Oberfläche der eingesetzten Materialien. Die gerichtete, kovalente Bindung auf der Mikropartikeloberfläche lies keine unspezifische Einzelstrang-DNS (ssDNS)-

Adhäsion im Vergleich zum ELISA zu. Demzufolge gab es keine Reaktionen von anti-ssDNS AAk mit der antigenbeladenen Mikropartikeloberfläche. Die Relevanz von anti-ssDNS AAk ist in der Diagnostik von SARE strittig. In hochtitriger Form können sie als zusätzlicher Indikator für den Medikamenten-induzierten Lupus fungieren. Das Vorkommen von AAK gegen ssDNS in weiteren Autoimmun- sowie Infektionserkrankungen und Entzündungsprozessen relativiert jedoch die Bedeutung und senkt die diagnostische Spezifität dieser AAk [10]. Demnach ist der Nachweis von reinen anti-dsDNS AAk als spezifischer einzustufen als der kombinierte Nachweis von AAK gegen ds- und ssDNS. In Übereinstimmung mit der hier durchgeführten Studie war dies anhand der ermittelten diagnostischen Spezifitäten, welche sich um 11,6% unterschieden, eindeutig.

4.2 Entwicklung eines Screeningtests zur Detektion von anti-GP2 AAk als neuen, sensitiven Marker in Patienten mit PSC

Zellbasierte Screeningteste sind durch ihr breites Spektrum an autoantigenen Zielstrukturen ein sehr sensitives Nachweisverfahren für die Diagnostik von AIE. Für die Entwicklung eines multiparametrischen Tests auf Basis der CytoBead Technologie wird ein sensitiver Screeningtest mit einem spezifischen Bestätigungstest vereinigt. In der vorliegenden Publikation Sowa *et al*. 2018 wurde der Screeningtest als wichtiger Bestandteil des Multiparametertests für die serologische Diagnostik von GP2 spezifischen AAk entwickelt. Speziell wurde der Fokus auf den Nachweis von anti-GP2 AAk in Patienten mit PSC gelegt. Neben der Zielrichtung der Multiparameter-Testentwicklung stand gleichauf die Fragestellung nach einem neuen serologischen Marker für Patienten mit PSC. Tatsächlich ist bislang kein spezifisches Autoantigen für die PSC im Gegensatz zu anderen autoimmunen Lebererkrankungen, wie zum Beispiel der AIH, beschrieben worden. Zwar zeigten kürzlich publizierte Studien ein vermehrtes Auftreten von atypischen, perinukleären ANCA in Patienten mit PSC, jedoch ist die Sensitivität bei nur etwa 30-40% zu finden und die Zielstruktur dieser ANCA unbekannt [31, 57]. Vor allem ANCA IgA wird mit der Zirrhose als Phänotyp der PSC in Verbindung gebracht. Darüber hinaus publizierten die Arbeitsgruppen von Papp 2015 und Jendrick 2017 die ersten Nachweise von anti-GP2 IgA in Patienten mit PSC, was die Grundlage für das Design und die Fragestellung dieser Studie darstellte [59, 60].

Für den Aufbau des Screening Tests wurde eine Transduktion von HEp-2 Zellen mit $GP2_{1-4+Leervektor}$ durchgeführt. Es konnte gezeigt werden, dass mit dem Zusatz des Glycosylphosphatidylinositol-Moleküls, $GP2_{1-4}$ membranständig, in nativer Konformation auf der Oberfläche der HEp-2 Zellen präsentiert wird. Der Nachweis erfolgte hierbei durch hausinterne, spezifische Antiseren. Die umfangreiche Evaluierungsstudie unterstützt die bisher veröffentlichten Daten zur Detektion eines neuen, sensitiven, serologischen Markers für Patienten mit PSC. Dies bedeutet einen möglichen Wandel in der serologischen

Diagnostik dieser autoimmunen Lebererkrankung. Im Speziellen zeigte IgA gegen GP2 Isoformen eine signifikant höhere Spezifität als IgG. Die höchste Diskriminierungsrate von PSC Patienten und getesteten Kontrollgruppen konnte durch die Nachweise von anti-$GP2_1$ IgA (47,2%) und anti-$GP2_4$ IgA (48,6%) erzielt werden. Wenn beide AAk-Nachweise miteinander kombiniert wurden, konnte eine signifikant höhere Sensitivität von 66,0% erzielt werden. Dies bedeutet gleichzeitig auch die höchste Prävalenz für den Nachweis von GP2-AAk IgA. Das Auftreten der jeweiligen Isoform-spezifischen AAk in Kombination mit der Präsenz der entsprechenden Ig-Klasse zeigte ebenfalls Korrelation zur phänotypischen Ausrichtung der Patienten mit PSC. Hierbei konnte gezeigt werden, dass anti-$GP2_{1+4}$ IgA ein möglicher positiver unabhängiger Prädiktor für Zirrhose ist. Weiterhin konnte anti-$GP2_2$ IgA als negativer unabhängiger Prädiktor für die Lebertransplantation identifiziert werden.
Schlussfolgernd scheint es möglich, für die serologische Diagnostik der PSC anti-GP2 IgA als neuen potentiellen Marker heranzuziehen. Aufgrund der signifikanten Assoziation von IgA gegen GP2 Isoformen mit dem Schweregrad der Erkrankung kann eine pathogenetische Rolle dieser IgA-AAk vermutet werden. Dies bleibt jedoch weiteren Untersuchungen vorbehalten und konnte im Rahmen dieser Dissertation nicht umfänglich bearbeitet werden. Die Entwicklung des Screeningtests als essentiellen Grundbaustein für die Neuentwicklung eines CytoBead zur serologischen Diagnostik von autoimmunen Lebererkrankungen konnte in diesem Rahmen erfolgreich absolviert werden.

Durch die erfolgreiche Entwicklung dieses Screeningstests konnte zusätzlich eine Basis für weitere Untersuchungen geschaffen werden. Eine weitere Studie befasste sich mit der Entwicklung und Charakterisierung von kameliden, rekombinanten Einzeldomänen-Antikörpern (VHHs). Die thermostabilen VHHs sind in der Lage spezifisch $GP2_{1-4}$ zu binden und werden anschließend mittels IIF nachgewiesen. Für den Einsatz in der serologischen Diagnostik von autoimmunen Lebererkrankungen können VHHs in der Zukunft zur Festphasenimmobilisierung von GP2 eine wichtige Rolle spielen [15].

Zusammenfassung

Die Zunahme der Komplexität diagnostischer Fragestellungen veranlasst die Entwicklung von innovativen Teststrategien. Multiparameterteste sind eine Antwort auf die sich im Wandel befindende Routinediagnostik von Autoimmunerkrankungen.

In der vorliegenden kumulativen Dissertation wurde eine neue multiparametrische Technologie für den Bereich der Autoimmundiagnostik entwickelt und klinisch evaluiert. Diese CytoBead Technologie ist eine Kombination aus Screeningtest, definiert durch die indirekte Immunfluoreszenz als diagnostischen Goldstandard, und einen multiplexen Bestätigungstest, welcher durch einen Mikropartikel-basierten Immunfluoreszenztest umgesetzt wurde.

Zur serologischen Diagnostik von anti-neutrophilen zytoplasmatischen Antikörpern (ANCA) assoziierten Vaskulitiden wurde der CytoBead ANCA entwickelt. Dieser Multiparametertest beinhaltet eine Kombination aus indirekter Immunfluoreszenz mit Ethanol fixierten Granulozyten und Mikropartikel-basiertem Immunfluoreszenztest mit Proteinase 3 und Myeloperoxidase. Wesentliche Vorteile gegenüber den klassischen Testen sind Zeit- und Kosteneinsparungen, die in der Routinediagnostik unerlässlich sind. Unter der Prämisse der deutlichen Zeiteinsparung von mehreren Bearbeitungsstunden oder sogar Tagen wurde diese Technologie für verschiedene, krankheitsspezifische Bereiche als Werkzeug der Profildiagnostik entwickelt.

Besonders im Bereich der Notfalldiagnostik von Autoimmunerkrankungen zählt jede Stunde, wenn es um das Überleben der Patienten geht. Die Weiterentwicklung dieser Technologie für die serologische Diagnostik der Rapid Progressiver Glomerulonephritis (RPGN) als Notfalldiagnostik, mit der Kombination aus indirekter Immunfluoreszenz mit Ethanol fixierten Granulozyten und Mikropartikel basiertem Immunfluoreszenztest mit Proteinase 3, Myeloperoxidase, Glomerulärer Basalmembran und doppelsträngiger Desoxyribonukleinsäure, ist ein wesentlicher Beitrag für eine effiziente *in-vitro* Diagnostik. Die sich anschließenden klinischen Studien des CytoBead ANCA und CytoBead RPGN wiesen gute bis sehr gute Vergleichbarkeiten zu klassischen Testen auf. Die Studien untermauerten zudem die Wichtigkeit der CytoBead Technologie hinsichtlich Nachhaltigkeit und Patientenmonitoring. Ein wichtiges Hauptaugenmerk der Testentwicklung lag in der Stabilität, Reproduzierbarkeit, Standardisierung und Quantifizierbarkeit von Ergebnissen, die im Rahmen dieser Dissertation evaluiert werden konnten. Mit einer digitalen, fluoreszenzbasierten Bildverarbeitungssoftware des AKLIDES® Systems konnten die Ergebnisse dieser Technologie zudem objektiviert werden.

Für eine weitere CytoBead Entwicklung und Evaluierung im Bereich der autoimmunen Lebererkrankungen konnte im Rahmen dieser Dissertation ein wichtiger Grundstein gelegt

werden. Es wurde ein Screeningtest auf Basis der indirekten Immunfluoreszenz mit Glykoprotein $2_{1,2,3,4}$ transduzierten HEp-2 Zellen entwickelt und anschließend in einer klinischen Studie evaluiert. Innerhalb dieser Studie konnte eine mögliche neue, autoantigene Zielstruktur für Patienten mit Primär Sklerodisierende Cholangitis identifiziert werden. Hauptsächlich IgA Autoantikörper gegen Glykoprotein $2_{1,4}$ scheinen pathognomonisch für die Primär Sklerodisierende Cholangitis zu sein. Zusätzlich konnte eine Korrelation des Phänotyps der Primär sklerodisierenden Cholangitis mit dem Auftreten von anti-Glykoprotein 2 IgA gezeigt werden, welche eine pathogenetische Rolle dieser Schleimhaut-assoziierten Autoantikörper vermuten lässt.

Zusammenfassend stellt sich die Entwicklung der CytoBead Technologie als eine neue, innovative Generation der serologischen Diagnostik von Autoimmunerkrankungen dar, welche zusätzlich auf verschiedene Diagnostikprofile angepasst werden kann.

Summary

The growing demand within the healthcare system for new innovative diagnostics needs to be addressed by the development of novel, innovative strategies. In case of *in-vitro* diagnostics for autoimmune disorders, a new era has been ushered in by the development of novel multiparametric assay techniques becoming part of the daily laboratory workflow.

In the current cumulative thesis a new multiparametric technology, named CytoBead, was developed and evaluated. It was established to have a highly variable tool for profiled serological diagnostics of autoimmune disorders. The CytoBead combines an indirect immunofluorescence screening method with a microbead immunoassay as confirmatory assay. For the serological diagnostics of anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis, the CytoBead ANCA assay combining ANCA testing with anti-proteinase 3 and anti-myeloperoxidase antibody microbead immunoassays was developed and subsequently evaluated within a clinical study. The main advantages of the new CytoBead assay in contrast to the classical assays are the time and cost efficiency.

Especially, in an autoimmune emergency case like the rapidly progressive glomerulonephritis (RPGN), fast and reliable diagnostic results are essential for the survival and the well-being of the patients. According to the required profile diagnostics of this disease, the CytoBead RPGN was developed. Thus, the assay contains ethanol fixed granulocytes as well as microbeads coated with proteinase 3, myeloperoxidase, doubled-stranded desoxy ribonucleic acid and glomerular basement membrane antigen. The clinical studies of the CytoBead ANCA and RPGN assay showed good to very good agreement with classical assays and provided important information regarding the assay characteristics, stability, standardization and performance. Using novel digital image processing software and the AKLIDES® system, a fully quantitative analysis of all results was obtained.

Another key aspect in the current work was the development and evaluation of a new indirect immunofluorescence assay for the serological diagnosis of primary sclerosing cholangitis by analysis of anti-Glycoprotein 2 autoantibodies. The assay was based on Glycoprotein 2 isoform-transduced HEp-2 cells. In the corresponding clinical study, a significant association of anti-Glycoprotein $2_{1,2,3,4}$ IgA with primary sclerosing cholangitis was revealed. Consequently, a potentially novel autoantigenic target was found for primary sclerosing cholangitis. Furthermore, a correlation of anti-Glycoprotein 2 IgA with the clinical phenotype in primary sclerosing cholangitis was determined, which could indicate a possible pathogenetic role of these autoantibodies. This screening assay was the basis for the development of a new CytoBead assay for the serological diagnosis of autoimmune liver diseases.

Summarizing the development of the novel CytoBead technology, this multiparametric assay is a new generation for autoantibody detection and applicable for different diagnostic profiles.

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Dokumentation / Publikationsliste

Die folgenden Publikationen sind Bestandteil der vorliegenden kumulativen Dissertation. Als Erstautor der Publikationen war ich für die Entwicklung der CytoBead Technologie und des Testformats der IIF, das Studiendesign, die Studiendurchführung, Datenerfassung, Auswertung und Einreichung sowie Korrekturen im Review Verfahren der jeweiligen Journals verantwortlich.

In der Publikation Sowa *et al*. 2018 gab es eine geteilte Erstautorenschaft, bei welcher Rafal Kolenda für die Transduktion der Zelllinie und Überprüfung des Transduktionserfolges mittels Durchflusszytometrie verantwortlich war. In dieser Publikation war ich für die Entwicklung des Testes im Testformat der IIF, das Studiendesign, die Studiendurchführung, Datenerfassung, Auswertung und Einreichung sowie Korrekturen im Review Verfahren des Journals verantwortlich.

- **Sowa M**, Grossmann K, Knütter I, Hiemann R, Röber N *et al.* (2014) SIMULTANEOUS AUTOMATED SCREENING AND CONFIRMATORY TESTING FOR VASCULITIS-SPECIFIC ANCA. PloS One):e107743

- **Sowa M**, Grossmann K, Scholz J, Röber N, Rödiger S *et al*. (2014) THE CYTOBEAD ASSAY – A NOVEL APPROACH OF MULTIPARAMETRIC AUTOANTIBODY ANALYSIS IN THE DIAGNOSTICS OF SYSTEMIC AUTOIMMUNE DISEASES / DER CYTOBEAD-ASSAY – EINE NEUE MÖGLICHKEIT DER MULTIPARAMETRISCHEN AUTOANTIKÖRPERANALYTIK BEI SYSTEMISCHEN AUTOIMMUNERKRANKUNGEN. J Lab Med 38(6):309-317

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(Erstautoren sind jeweils unterstrichen; Belegexemplare etwaiger Vorveröffentlichungen befinden sich im Anhang)

Buchbeiträge

- **Sowa M**, Knütter I, Hiemann R, Grossmann K, Röber N *et al*. CYTOBEAD ANCA - A NOVEL INDIRECT IMMUNOFLUORESCENCE TEST FOR THE SIMULTANEOUS DETECTION OF ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODIES. (2013) Buch: Conrad K, Chan EKL, Fritzler MJ, Humbel RL, Meroni PL, Steiner G, Shoenfeld Y. INFECTION, TUMORS AND AUTOIMMUNITY, Report of the 11th Dresden Symposium on Autoantibodies. Methodical Aspects and Diagnostic Strategies, Vol 9 – 2013, Pabst Science Publisher Seite 271, ISBN 978-3-89967-881-9

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Weitere Beiträge

- 02.09.2013 Fachvortrag und Posterpräsentation zum 11. Dresden Symposium on Autoantibodies in Dresden „CytoBead ANCA – a novel indirect immunofluorescence test for simultaneous detection of anti-neutrophil cytoplasmic antibodies

- 18.04.2013 Fachvortrag zum 7. Senftenberger Innovationsforum Multiparameteranalytik in Senftenberg „Simultaneous detection of anti-neutrophil cytoplasmic antibodies and reactivity to corresponding antigenic targets by a novel indirect immunofluorescence test“

- 13.05.2014 Gewinnerin des XII BIONNALE Speed Lecture Awards 2014 in Berlin „CytoBead ANCA Test – Neue Diagnostik für Notfälle“

- 23.06.2014 Fachvortrag und Poster zum IFCC Worldlab in Istanbul (Türkei) „A novel CytoBead Immunoassay for simultaneous detection of celiac-disease specific antibodies and total IgA“

- 19.-22.09.2014 Posterpreis zum Deutschen Kongress für Laboratoriumsmedizin in Mannheim „SIMULTANEOUS SCREENING AND CONFIRMATION OF ANCAS AND DETECTION OF ANTI-GBM ANTIBODIES IN CASE OF EMERGENCY“

- 19.-22.04.2015 Poster zum 17. Internationalen Vaskulitis & ANCA Workshop in London (UK) „Unique Technology for the simultaneous Screening and Confirmation

of Autoantibodies in Emergency Situations of Rapidly Progressive GlomeruloNephritis“

- 09.04.2016 und 08.04.2016 Fachvorträge zum 10. International Congress on Autoimmunity in Leipzig „Next generation autoantibody detection by highly flexible CytoBead® technology“ und „Combined determination of autoimmune liver disease specific autoantibodies by CytoBead® assay“

- 29.09.2017 Fachvortrag zum 13. Dresden Symposium on Autoantibodies in Dresden „Mucosal Autoimmunity to cell-bound GP2 isoforms 1 and 4 is a sensitive marker in severe Primary Sclerosing Cholangitis“

- 17.05.2018 Fachvortrag zum 11. International Congress on Autoimmunity in Lissabon (Portugal) „Mucosal Autoimmunity to cell-bound GP2 isoforms 1 and 4 is a sensitive marker in severe Primary Sclerosing Cholangitis“

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Anhang

Im Folgenden befinden sich die Belegexemplare etwaiger Vorveröffentlichungen.

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Simultaneous Automated Screening and Confirmatory Testing for Vasculitis-Specific ANCA

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Abstract

Anti-neutrophil cytoplasmic antibodies (ANCA) are the serological hallmark of small vessel vasculitis, so called ANCA-associated vasculitis. The international consensus requires testing by indirect immunofluorescence (IIF) on human ethanol-fixed neutrophils (ethN) as screening followed by confirmation with enzyme-linked immunosorbent assays (ELISAs). This study evaluates the combination of cell- and microbead-based digital IIF analysis of ANCA in one reaction environment by the novel multiplexing CytoBead technology for simultaneous screening and confirmatory ANCA testing. Sera of 592 individuals including 118 patients with ANCA-associated vasculitis, 133 with rheumatoid arthritis, 49 with infectious diseases, 77 with inflammatory bowel syndrome, 20 with autoimmune liver diseases, 70 with primary sclerosing cholangitis and 125 blood donors were tested for cytoplasmic ANCA (C-ANCA) and perinuclear ANCA (P-ANCA) by classical IIF and ANCA to proteinase 3 (PR3) and myeloperoxidase (MPO) by ELISA. These findings were compared to respective ANCA results determined by automated multiplex CytoBead technology using ethN and antigen-coated microbeads for microbead immunoassays. There was a good agreement for PR3- and MPO-ANCA and a very good one for P-ANCA and C-ANCA by classical and multiplex analysis (Cohen's kappa [κ] = 0.775, 0.720, 0.876, 0.820, respectively). The differences between classical testing and CytoBead analysis were not significant for PR3-ANCA, P-ANCA, and C-ANCA ($p<0.05$, respectively). The prevalence of confirmed positive ANCA findings by classical testing (IIF and ELISA) compared with multiplex CytoBead analysis (IIF and microbead immunoassay positive) resulted in a very good agreement (κ = 0.831) with no significant difference of both methods (p = 0.735). Automated endpoint-ANCA titer detection in one dilution demonstrated a very good agreement with classical analysis requiring dilution of samples (κ = 0.985). Multiplexing by CytoBead technology can be employed for simultaneous screening and quantitative confirmation of ANCA. This novel technique provides fast and cost-effective ANCA analysis by automated digital IIF for the first time.

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Introduction

Autoimmune vascular disorders comprising granulomatosis with polyangiitis (GPA, formerly Wegener's granulomatosis), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA, formerly Churg-Strauss syndrome) are characterized by microvascular inflammation, tissue necrosis, and the appearance of anti-neutrophil cytoplasmic antibody (ANCA) [1–6]. Thus, the term ANCA-associated vasculitis has been coined for this distinct disease group characterized by loss of tolerance to neutrophilic targets. According to the international consensus statement for ANCA testing, indirect immunofluorescence (IIF) findings on ethanol-fixed human neutrophils (ethN) are recommended to be confirmed with antigen-specific enzyme-linked immunosorbent assays (ELISAs) [2,4,7,8]. ANCA IIF reveals two main patterns on ethN sub-classifying ANCAs into cytoplasmic ANCA (C-ANCA) and perinuclear ANCA (P-ANCA). The C- and P-ANCA in human patients with ANCA-associated vasculitis are mainly directed against proteinase 3 (PR3) and myeloperoxidase (MPO), respectively, and seem to be associated with disease activity [9,10]. However, ANCA IIF patterns as well as PR3- and MPO-ANCA can be observed in other inflammatory conditions

and several ANCA-specific targets apart from MPO and PR3 have been reported which lowers the specificity of ANCA testing by IIF [11,12]. Thus, a C-ANCA pattern confirmed by PR3-ANCA ELISA positivity is indicative for GPA [1,3], whereas a P-ANCA pattern confirmed by a positive MPO-ANCA ELISA finding supports the diagnosis of MPA and EGPA [11]. Furthermore, the corresponding ANCA titers are often associated with activity of disease in patients with GPA and MPA.

Consequently, appropriate ANCA testing requires two independent assay techniques to be run currently. Thus, the combination of both IIF and antigen-specific assays was confirmed in several studies to be the optimal strategy for ANCA detection [13].

Recently, IIF microscopy employing fluorescent microbeads as solid phase has been reported offering the opportunity to multiplex autoantibody analysis [14,15]. For the first time, we employed this novel multiplexing technique along with ethN-based IIF for the development of one reaction environment to combine screening and confirmatory ANCA testing. Thus, pattern recognition of P-ANCA and C-ANCA on ethN was aligned with the quantitative determination of PR3- and MPO-ANCA by the means of a novel software module for the automated pattern recognition system Aklides. Existing multiplex ANCA testing such as the mosaic technique does not offer these benefits [16].

Automated digital IIF has been used in HEp2-cell based assays for analysis of antinuclear (ANA) and dsDNA antibodies. Moreover, analysis of respective autoantibody endpoint titers without serial dilution became available by the introduction of calibration tools for digital immunofluorescence [17–22]. We developed a similar technique for ANCA-endpoint titer determination by the novel combined ANCA test. Thus, the novel CytoBead test system presents a unique combination of a classical cell-based assay with multiplexing microbead technology for the simultaneous quantitative analysis of ANCA and their specificities to PR3 and MPO.

In the present study, we evaluated the performance of the novel CytoBead ANCA assay and compared it with classical ANCA testing by independent techniques. Furthermore, we compared the quantitative assessment of PR3- and MPO-ANCA as well as the ANCA-endpoint-titer analysis of the CytoBead ANCA assay on the automated interpretation system Aklides with classical ELISA and IIF methods.

Materials and Methods

Patients and controls

Sera of 592 individuals including 118 patients with ANCA-associated vasculitis, 300 with autoimmune and gastrointestinal diseases, 49 with infectious diseases, and 125 blood donors (BD) were enrolled for the present evaluation (Table 1; patient sera are non-consecutive). Patients with ANCA-associated vasculitis were diagnosed based on typical disease history, characteristic clinical findings, and confirmed clinical histology according to the criteria of the 1994 Chapel Hill Consensus Conference, the consensus statement of 1999, 2012 and the 1990 American College of Rheumatology [2,4,23]. Serum samples were obtained from patients with a confirmed clinical diagnosis of GPA or MPA irrespective of serology.

Serum samples from patients with rheumatoid arthritis (RA), primary sclerosing cholangitis (PSC), autoimmune hepatitis type1 and 2, ulcerative colitis (UC), Crohn's disease (CD) were used as disease controls (Table 1). In total, 49 sera from patients with infectious disease (cytomegalovirus [CMV], rubella virus, Toxoplasma gondii, Epstein-Barr virus [EBV]) were included as further disease controls. In particular, patients with herpes viral infections have the potential to induce ANCA production due to overall B cell stimulation and, thus, could demonstrate false-positive results.

The study received approval from the ethical committee of the Technical University of Dresden (EK56022014) and fulfilled the ethical guidelines of the most recent declaration of Helsinki. An approval of the donors was not necessary because fully anonymized probes used as quality controls in routine diagnostics were selected for this study only. The ethical committee waived the need for written informed consent from the participants accordingly.

Detection of PR3- and MPO-ANCA with antigen-specific ELISA

PR3- and MPO-ANCA were detected using commercially available antigen-specific ELISAs according to instructions of the manufacturers (GA Generic Assays GmbH, Dahlewitz, Germany; Orgentec GmbH, Wiesbaden, Germany) as described elsewhere [24,25]. The PR3- and MPO-ANCA ELISAs of GA Generic Assays GmbH revealed intra-assay variabilities of 5.2% each and inter-assay variabilities of 6.2% each for a serum with 20.0 U/mL PR3-ANCA and 20.0 U/mL MPO-ANCA, respectively. The PR3- and MPO-ANCA ELISAs of Orgentec GmbH revealed intra-assay variabilities of 3.3% and 4.1% for sera with 14.0 U/mL PR3-ANCA and 30.2 U/mL MPO-ANCA and inter-assay variabilities of 6.8% and 4.9% for sera with 51.7 U/mL PR3-ANCA and 33.8 U/mL MPO-ANCA, respectively.

Determination of ANCA by indirect immunofluorescence

PR3- and MPO-ANCA have been analyzed by IIF employing a commercial kit with ethN (GA Generic Assays GmbH). Patient sera and control sera were diluted 1/20 and 50 µl per well were used. The sera were incubated for 30 minutes on the slides and afterwards washed five times each two minutes with phosphate buffered saline (PBS). Subsequently an AlexaFluor 488 conjugated polyclonal anti-human IgG antibody (Dianova GmbH, Hamburg, Germany) was used as secondary antibody and incubated again 30 minutes. After incubation the slides were washed accordingly and the wells were covered with a specific covering solution. The slides were evaluated automatically using the Aklides platform (Medipan, Berlin/Dahlewitz, Germany) as described elsewhere [26]. Briefly, images were assessed automatically using a motorized inverse microscope (IX81, Olympus Corporation, Tokyo, Japan) with a motorized scanning stage (IM120, Märzhäuser, Wetzlar, Germany); 400 nm and 490 nm light-emitting diodes (LED) (PrecisExcite, CoolLED, Andover, UK), and a charge-coupled device grey-scale camera (DX4, Kappa, Gleichen, Germany). The interpretation system is controlled by the Aklides software consisting of modules for device and autofocus control, image analysis, and pattern recognition algorithms. The novel autofocus based on Haralick's image characterization of objects through grey-scale transition using DAPI as fluorescent dye for focusing, quality evaluation, and object recognition. Two-dimensional images were acquired using an objective with 40-fold magnification (Olympus semiapochromat LUCPLFLN 40X, 0.60 NA, W.D. 2.7–4.0 mm). Fluorescence detection was performed using LED excitation with appropriate multiband filter for the DAPI and FITC dyes (DA/FI-A, Semrock, Rochester, USA). Single DAPI and FITC image were serially captured and stored in lossless compressed Tagged Image File (TIF) format.

Table 1. Characteristics of patients and controls: 118 patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis, 300 with autoimmune and gastrointestinal diseases, 49 with infectious disorders, and 125 blood donors (BD) were enrolled in the study.

Diagnosis	N	Gender	Age	Age
		f/m	median	interquartile range
ANCA-associated vasculitis				
GPA	90	51/39	65	56–89
MPA	28	14/14	67	51–72
Autoimmune disease controls				
RA	133	99/34	62	56–69
PSC	70	21/49	45	35–57
AIH I	10	8/2	13	12–15
AIH II	10	10/0	11	8–14
UC	57	31/26	49	38–57
CD	20	15/5	40	32–54
Infectious disease controls				
Toxoplasmosis	16	15/1	34	27–43
CMV	25	23/2	38	33–41
Rubella	5	5/0	36	31–37
EBV	3	2/1	10	7–23
Blood Donors	125	64/61	21	21–26

AIH, autoimmune hepatitis; CMV, Cytomegalovirus; CD, Crohn's disease; EBV, Epstein-Barr virus; f, female; GPA, granulomatosis with polyangiitis; m, male; MPA, microscopic polyangiits; N, number; PSC, primary sclerosing cholangitis; RA, rheumatoid arthritis; UC, ulcerative colitis.
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Multiplex detection of ANCA by CytoBead ANCA

CytoBead ANCA (GA Generic Assays GmbH) is a multiplex IIF test in one reaction environment combining the screening of ANCA on ethN and their confirmation with multiplex microbead immunoassays, using 9 μm and 15 μm red fluorescent microbeads (excitation 610 nm/emission 690 nm) coated with recombinant antigens PR3 and MPO, respectively. Triple parted wells on microscopic glass slides were employed for the fixation of neutrophils in the middle compartment as well as PR3- and MPO-coated microbeads in the right compartment (Fig. 1). The left compartment was not used and can be employed for further antibody determinations in the framework of an autoantibody profiling [27,28]. Furthermore, a reference microbead population of 12 μm labelled by a green emitting fluorescence dye filling the entire microbead is immobilized on the right compartment. Thus, the differently sized green fluorescence halos of positively stained PR3- and MPO-coated microbeads can be distinguished. In general, PR3-ANCA positive sera show cytoplasmic fluorescence patterns on ethN and a green fluorescence halo on the surface of PR3-coated microbeads only. In contrast, MPO-ANCA positive sera show perinuclear fluorescence patterns on ethN and a green fluorescence halo on the surface of MPO-coated microbeads. For automation, the fluorescence intensities of the fluorescence halos can be quantified and simultaneously located to the appropriate microbead population by the Aklides system.

Fully automated interpretation and pattern recognition of ANCA

The concept of the fully automated interpretation system Aklides for evaluation of ANCA IIF patterns is based on novel mathematical software algorithms for pattern recognition [25,26]. To obtain a reproducible IIF read out signal, the excitation light intensity was calibrated employing a recently developed calibration tool [20]. Novel fluorescent calibration microbeads employed guarantee satisfactory inter-laboratory reproducibility for the calibration process.

Cells and microbeads were characterized by regional, topological, and texture/surface descriptors by employing image data of DAPI and FITC for cells and Cy5 for microbeads. A minimum of 20 stained ethN and 50 microbeads were counted at each sample. The obtained mean fluorescence intensities (MFI) reflect the specific ANCA reactivity of the serum sample. The final read out is expressed as arbitrary units.

Automated endpoint-titer ANCA determination with Aklides

Automated ANCA endpoint-titer determination avoiding serial dilution of samples was developed using analysis algorithms for endpoint-titers of antinuclear antibodies described recently. In order to compensate the different MFI of the two ANCA patterns, the Aklides software harmonizes the fluorescence intensity measurement by including several object description characteristics for MFI analysis. Nevertheless, ANCA positive sera with differing classical end-point titers diluted at 1 to 20 revealed differing MFI values in particular for higher titers depending on the ANCA pattern. Thus, the novel software module for ANCA end-point titer determination employs different algorithms depending on the ANCA IIF pattern analysed by the Aklides system.

Quantification of ANCA with lot-specific standard curves

For the quantification of ANCA by microbead immunoassays of CytoBead testing, computer-stored lot-specific standard master curves were established. Stable microbead reactivity permitted the use of a single lot-specific standard curve to quantify ANCA

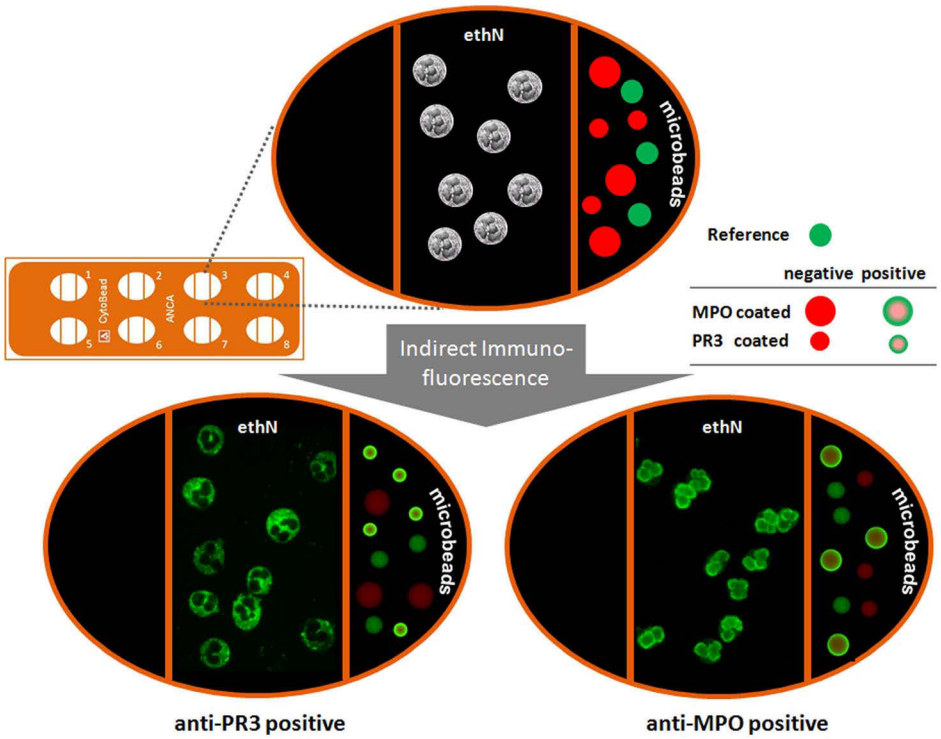

Figure 1. CytoBead ANCA assay principle. Microscopic glass slides with ethanol-fixed human neutrophils (ethN; middle compartment of the well) and proteinase 3 (PR3) as well as myeloperoxidase (MPO) coated microbeads (right compartment of the well) are used for detection of anti-neutrophil cytoplasmic antibodies (ANCAs) by ethN-based indirect immunofluorescence and simultaneous analysis of PR3- and MPO-ANCA by microbead immunoassay. PR3-ANCA positive sera show cytoplasmic fluorescence patterns on ethN and a green fluorescence halo on the surface of PR3-coated microbeads (9 µm). In contrast, MPO-ANCA positive sera show perinuclear fluorescence patterns on ethN and a green fluorescence halo on the surface of MPO-coated microbeads (15 µm).
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concentrations. Thus, these standard master curves were obtained by assaying dilutions (1/20 to 1/2560) of reference sera for MPO-ANCA (human reference serum #15) and PR3-ANCA (human reference serum #16) of the International Center for Disease Control and Prevention (CDC).

After acquisition by the Aklides system, PR3- and MPO-ANCA standard curves were fitted using a 5-parameter logistic-fitting curve model [29]. Curve fit parameters were then stored in a post-analysis charge certificate and provided for each assay run to analyze the obtained MFI data. Quantitative data are processed after recalibration of initial stored lot-specific master curves by a two-point recalibration using adjuster signal levels of the current run.

For assay performance assessment, intra- and inter-assay coefficients of variations (CV) were calculated by a eight-fold measurement of serum samples within one run (intra-assay) and further by measurement on 3 different days (inter-assay). The functional assay sensitivity (limit of quantification), being the lowest detectable concentration with an inter-assay CV lower or equal than 20%, for PR3- and MPO-ANCA was determined as described previously [30].

Data standardisation

For the data comparison, ELISA findings in units per millilitre (U/ml) and CytoBead ANCA assay data in international units (IU) were standardized. The highest standard curve concentration points of the ELISA and CytoBead ANCA assay were referred to as 100% and results converted respectively.

Statistical analysis

Inter-rater agreement statistics (Cohen's kappa, κ) and McNemar's test were used for group comparison. P values below 0.05 were considered to be significant. Receiver operating characteristics (ROC) curve analysis was performed using MedCalc software (MedCalc, Mariakerke, Belgium; Version 12.4.0).

Results

CytoBead ANCA cut-off determination

To determine the cut-off of the novel CytoBead ANCA for PR3- and MPO-ANCA, 465 human sera of patients and controls including 118 patients with ANCA-associated vasculitis, 133 with RA, 49 with ID, 20 with CD, 20 with AIH and 125 BD were run with the Aklides IIF interpretation system. Patients with PSC and UC were excluded from the ROC curve analysis due to the known frequent number of positive ANCA (especially PR3-ANCA) findings in these patient groups. The obtained MFIs were standardised as described in Material and Methods and subjected to ROC curve analysis to obtain the respective cut-off values for each ANCA specificity (Fig. 2). For PR3-ANCA the calculated cut-off was 8.4% (0.9 IU/mL) and for MPO-ANCA 19.3% (3.0 IU/mL). The area under curve (AUC) was determined for PR3-ANCA employing 90 sera of patients with GPA as positive criterion at 0.896 (95% confidence interval [CI]: 0.864–0.923) and for MPO-ANCA using 28 patients with MPA as positive criterion at 0.934 (95% CI: 0.904–0.957); $p<0.0001$, respectively.

The PR3- and MPO-ANCA microbead immunoassays of the multiplex CytoBead testing displayed an intra-assay variability of

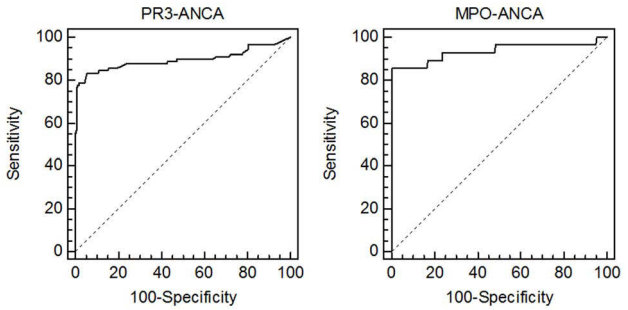

Figure 2. Receiver-operating characteristic curve analysis of anti-neutrophil cytoplasmic antibodies (ANCAs) to proteinase 3 (PR3) and myeloperoxidase (MPO) by CytoBead ANCA. 465 sera from 118 patients with ANCA-associated vasculitis, 133 with rheumatoid arthritis, 49 with infectious diseases, 20 with Crohn's disease, 20 with autoimmune hepatitis and 125 blood donors were included. PR3- and MPO-ANCA were determined simultaneously by microbead immunoassay employing 90 patients with granulomatosis with polyangiitis and 28 patients with microscopic polyangiitis as positive criterion, respectively.
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7.1% and 7.7% and an inter-assay variability of 7.2% and 7.6% for sera with 25 IU/mL PR3-ANCA and 100 IU/mL MPO-ANCA, respectively.

The functional assay sensitivity for PR3-ANCA and MPO-ANCA were analyzed as 5.8% (0.6 IU/ml) and 16.1% (2.5 IU/ml), respectively.

Comparison of ANCA prevalences determined by classical and multiplex CytoBead assays

ANCA immunofluorescence pattern as well as PR3- and MPO-ANCA were determined by classical ELISA and IIF and compared with respective findings by automated IIF and microbead immunoassay employing the CytoBead technology (Table 2). According to inter-rater agreement statistics, there was a good agreement for PR3- and MPO-ANCA ($\kappa = 0.775$, 95% CI: 0.710–0.839; 0.720, 95% CI: 0.596–0.843, respectively, Table 3). The agreement for P-ANCA and C-ANCA between classical IIF and CytoBead analysis was very good ($\kappa = 0.876$, 95% CI: 0.812–0.940; 0.820, 95% CI: 0.755–0.844, respectively).

The CytoBead technique determined one C-ANCA and one P-ANCA positives more in patients with GPA and MPA, respectively, compared with the classical method.

However, according to McNemar's test, the differences between classical testing and CytoBead analysis were not significant for PR3-ANCA, P-ANCA, and C-ANCA (1.18%, 95% CI: −1.14%–3.34%; 0.17%, 95% CI: −0.41%–0.50%; 0.34%, 95% CI: −1.25%–1.82%; $p<0.05$, respectively).

In contrast, MPO-ANCA demonstrated a significant difference for both methods (1.69%, 95% CI: 0.14%–2.65%; $p = 0.031$). Whereas there was no significant difference for positive MPO-ANCA findings obtained by both methods in controls, a tendency for a higher prevalence of positive MPO-ANCA detected by CytoBead microbead immunoassay (35/118, 29.7%) compared to those by EIA (23/118, 19.5%) was found in patients with ANCA-associated vasculitis. The CytoBead microbead immunoassay detected significantly more MPO-ANCA positives in patients with GPA in contrast to the classical ELISA (11/90, 12.2% *vs* 3/90. 3.3%, $p = 0.048$).

For the serological diagnosis of ANCA-associated vasculitis, a positive ANCA finding by IIF should be confirmed by a positive PR3- or MPO-ANCA result. Thus, we compared the prevalences of confirmed positive ANCA findings by classical testing (IIF and ELISA positive) with multiplex CytoBead analysis (IIF and microbead immunoassay positive) resulting in a very good agreement for both techniques ($\kappa = 0.831$, 95% CI: 0.777–0.885). McNemars test did not reveal a significant difference for confirmed positive ANCA findings obtained by classical and multiplex analysis (0.51%, 95% CI: −1.58%–2.50%; $p = 0.735$).

Furthermore, we compared the prevalences of positive C-ANCA findings by classical IIF testing confirmed by PR3 ANCA ELISA with respective multiplex CytoBead analysis (PR3 ANCA IIF and PR3 ANCA microbead immunoassay positive). There was a very good agreement for both techniques ($\kappa = 0.937$, 95% CI: 0.893–0.980) and McNemar's test did not reveal a significant difference (1.01%, 95% CI: −0.07%–1.34%; $p = 0.070$). The respective comparison of the prevalences for positive P-ANCA findings by classical IIF testing confirmed by MPO ANCA ELISA with multiplex CytoBead analysis (MPO ANCA IIF and MPO ANCA microbead immunoassay positive) revealed also a very good agreement for both techniques ($\kappa = 0.884$, 95% CI: 0.792–0.976). McNemar's test did not demonstrate a significant difference (0.34%, 95% CI: −0.56%–0.92%; $p = 0.688$).

Comparison of ANCA levels determined by classical and multiplex CytoBead assays

For the sake of comparison of ANCA levels by classical and multiplex testing, concentrations obtained by the different PR3- and MPO-ANCA assays were harmonized by standardizing values to the cut-offs of the respective assays and reporting them in % (Fig. 3). Standardized data of classical ELISA and multiplex testing by CytoBead microbead immunoassay were subjected to ROC curve analysis using 118 samples of patients with ANCA-associated vasculitis as disease criterion for ANCA *vs* 474 controls (Fig. 4). The AUC for PR3-ANCA by ELISA and microbead immunoassay did not demonstrate a statistical difference ($p>0.05$). In contrast, there was a significant higher AUC for MPO-ANCA levels by microbead immunoassay compared with those by ELISA ($p = 0.016$).

Automated endpoint-ANCA titer evaluation

Employing a test set of 34 sera from patients with GPA ($n = 8$), MPA ($n = 10$), RA ($n = 6$), and BD ($n = 10$), respective MFI values obtained by 1 to 20 diluted samples by CytoBead technology were compared with classical ANCA-endpoint titers determined by serial dilution in classical IIF. The resulting interdependence of classical P-ANCA and C-ANCA endpoint titers with quantitative MFI obtained by digital IIF were used to establish an automated method for the determination of endpoint ANCA titers in one dilution. Inter-rater agreement statistics revealed a very good agreement comparing both methods for endpoint ANCA titer analysis including all data pairs of titers equal or higher than 10 and combining titers equal or higher than 320 in a 6×6 frequency table (weighted $\kappa = 0.985$, 95% CI 0.980–0.991). In routine IIF autoantibody testing, a difference of one titer is not considered significant [31]. Accordingly, automated endpoint ANCA titer analysis of 586 (99.0%) out of the 592 samples investigated did not reveal different titers compared to those detected by the classical method (Table 4). Only 6 (0.1%) sera demonstrated differences of more than one ANCA titer level.

Discussion

For more than 25 years, ANCA serology has been an essential diagnostic tool for the differential diagnosis of vasculitic disorders and IIF is still considered the gold standard for ANCA screening

Table 2. Assessment of anti-neutrophil cytoplasmic antibodies (ANCA) and ANCA against proteinase 3 (PR3) and myeloperoxidase (MPO) by classical and automated multiplex analysis in one reaction environment.

	Classical testing					**CytoBead assay**				
	ELISA		*IIF*	*ELISA or IIF*	*ELISA and IIF*	*MIA*		*IIF*	*MIA or IIF*	*MIA and IIF*
samples (N)	*PR3-ANCA*	*MPO-ANCA*	*C/P-ANCA*			*PR3-ANCA*	*MPO-ANCA*	*C/P-ANCA*		
GPA (90)	78 (86.7%)	3* (3.3%)	77 (85.6%)	81 (90.0%)	77 (85.6%)	70 (77.8%)	11* (12.2%)	83 (92.2%)	85 (94.4%)	72 (80.0%)
MPA (28)	0 (0.0%)	20 (71.4%)	26 (92.9%)	26 (92.9%)	20 (71.4%)	0 (0.0%)	24 (85.7%)	28 (100.0%)	28 (100.0%)	24 (85.7%)
RA (133)	0 (0.0%)	0 (0.0%)	47 (35.3%)	47 (35.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	47 (35.3%)	47 (35.3%)	0 (0.0%)
PSC (70)	31 (44.3%)	3 (4.3%)	49 (70.0%)	53 (75.7%)	27 (38.6%)	24 (34.3%)	1 (1.4%)	49 (70.0%)	52 (74.3%)	21 (30.0%)
AIH1 (10)	3 (30.0%)	1 (10.0%)	9 (90.0%)	9 (90.0%)	1 (10.0%)	1 (10.0%)	1 (10.0%)	9 (90.0%)	9 (90.0%)	2 (20.0%)
AIH2 (10)	1 (10.0%)	1 (10.0%)	3 (30.0%)	3 (30.0%)	2 (20.0%)	2 (20.0%)	1 (10.0%)	3 (30.0%)	3 (30.0%)	3 (30.0%)
UC (57)	10 (17.5%)	1 (1.8%)	26 (45.6%)	29 (50.9%)	8 (14.0%)	18 (31.6%)	1 (1.8%)	26 (45.6%)	33 (57.9%)	10 (17.5%)
CD (20)	0 (0.0%)	0 (0.0%)	2 (10.0%)	2 (10.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (10.0%)	2 (10.0%)	0 (0.0%)
ID (49)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
BD (125)	0 (0.0%)	0 (0.0%)	7 (5.6%)	7 (5.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	13 (10.4%)	13 (10.4%)	0 (0.0%)

ANCA were determined by enzyme-linked immunosorbent assay (ELISA), classical indirect immunofluorescence (IIF) on ethanol-fixed neutrophils and multiplex CytoBead assay in patients and controls: 118 patients with ANCA-associated vasculitis, 300 with autoimmune and gastrointestinal diseases, 49 with infectious disorders, and 125 blood donors (BD).

* $p<0.05$.

AIH, autoimmune hepatitis; BD, blood donors; CD, Crohn's disease; c/p ANCA, cytoplasmic/perinuclear ANCA; GPA, granulomatosis with polyangiitis; ID, infectious diseases; MIA, microbead immunoassay; MPA, microscopic polyangiitis; N, number; PSC, primary sclerosing cholangitis; RA, rheumatoid arthritis; UC, ulcerative colitis.

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Table 3. Comparison of perinuclear (P-ANCA) and cytoplasmic (C-ANCA) anti-neutrophil cytoplasmic antibodies and ANCA against proteinase 3 (PR3) and myeloperoxidase (MPO) levels by classical and automated multiplex microbead assay analysis in one reaction environment.

PR3-ANCA		**CytoBead**			**C-ANCA**		**CytoBead**		
		positive	**negative**	**N**			**positive**	**negative**	**N**
ELISA	Positive	98	25	123	**Classical IIF**	positive	80	9	89
	Negative	18	451	469		negative	10	493	503
	N	116	476	592		N	90	502	592
MPO-ANCA		**CytoBead**			**P-ANCA**		**CytoBead**		
		positive	**negative**	**N**			**positive**	**negative**	**N**
ELISA	Positive	25	4	29	**Classical IIF**	positive	75	1	76
	Negative	14	549	563		negative	2	514	516
	N	39	553	592		N	77	515	592
		CytoBead (MIA or IIF)					**CytoBead (MIA and IIF)**		
		positive	**negative**	**N**			**positive**	**negative**	**N**
Classical testing	Positive	249	9	258	**Classical testing**	positive	116	19	457
(ELISA or IIF)	Negative	25	309	334	**(ELISA and IIF)**	negative	16	441	135
	N	274	318	592		N	460	132	592
PR3 ANCA		**CytoBead (PR3 ANCA MIA and C-ANCA IIF)**			**MPO ANCA**		**CytoBead (MPO ANCA MIA and P-ANCA IIF)**		
		positive	**negative**	**N**			**positive**	**negative**	**N**
Classical testing	Positive	68	7	75	**Classical testing**	positive	24	2	26
(PR3 ANCA ELISA and C-ANCA IIF)	Negative	1	516	517	**(MPO ANCA ELISA and P-ANCA IIF)**	negative	4	562	566
	N	69	523	592		N	28	564	592

PR3- and MPO-ANCA were determined by enzyme-linked immunosorbent assay (ELISA) and multiplex CytoBead microbead assay in 118 patients with ANCA-associated vasculitis, 300 with autoimmune and gastrointestinal diseases, 49 with infectious disorders, and 125 blood donors (BD).

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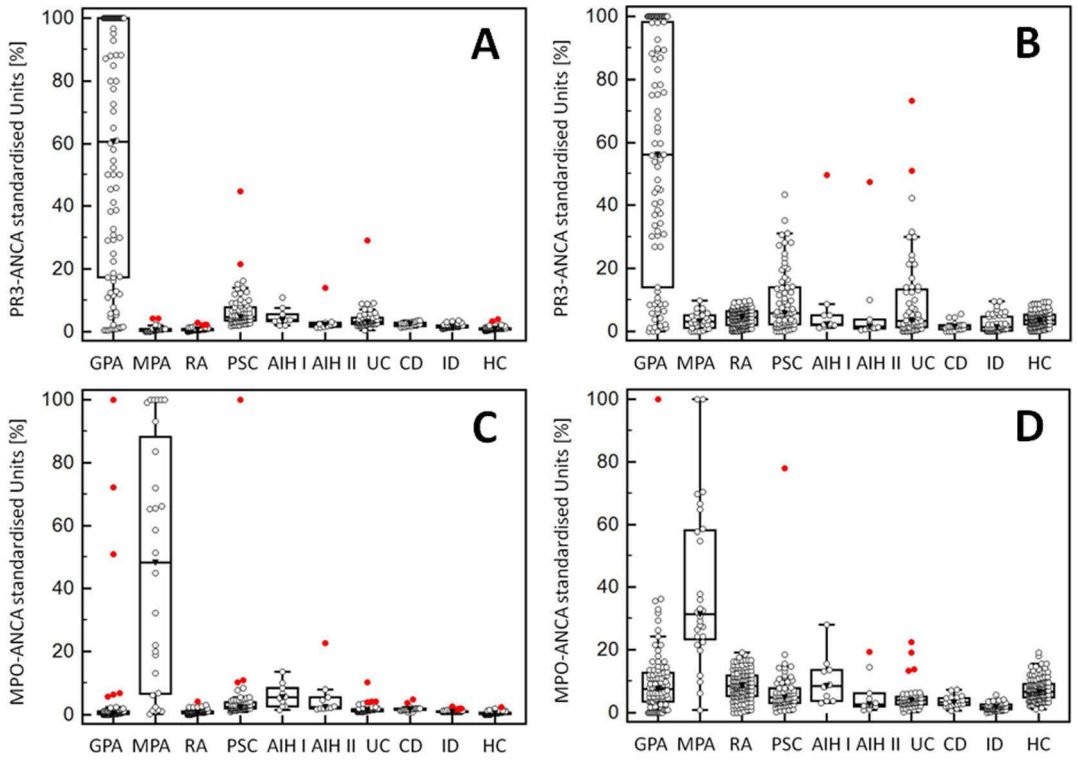

Figure 3. Anti-neutrophil cytoplasmic antibodies (ANCA) against proteinase 3 (PR3) and myeloperoxidase (MPO) levels by classical and automated multiplex microbead assay analysis in one reaction environment. PR3- and MPO-ANCA were determined by enzyme-linked immunosorbent assay (ELISA) (A, C, respectively) and multiplex CytoBead microbead assay (B, D, respectively) in 118 patients with ANCA-associated vasculitis, 300 with autoimmune and gastrointestinal diseases, 49 with infectious disorders, and 125 blood donors (BD). (Data are displayed in Box-and-Whisker plots with *far out* values, defined as values that are smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range, displayed as red circles.). AIH, autoimmune hepatitis; CD, Crohn's disease; GPA, granulomatosis with polyangiitis; ID, infectious diseases; MPA, microscopic polyangiitis; PSC, primary sclerosing cholangitis; RA, rheumatoid arthritis; UC, ulcerative colitis.
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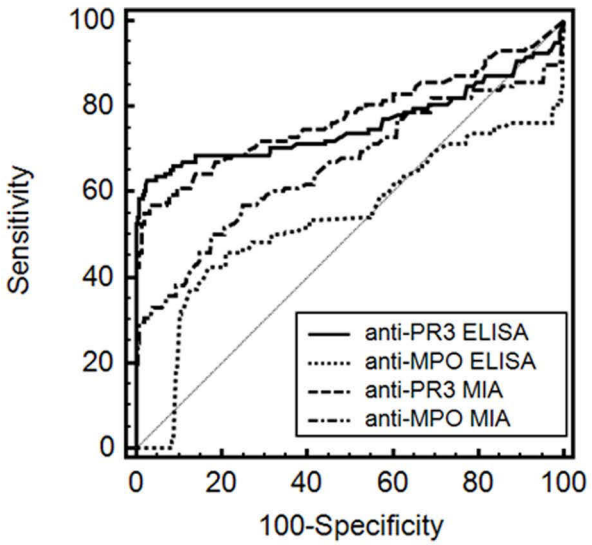

		ELISA	MIA
PR3-ANCA	AUC	0.758	0.776
	95% CI	0.722-0.792	0.74-0.809
	p-value	0.438	
MPO-ANCA	AUC	0.577	0.661
	95% CI	0.495-0.577	0.621-0.699
	p-value	0.016	

Figure 4. Receiver-operating characteristic curve analysis of anti-neutrophil cytoplasmic antibodies (ANCA) against proteinase 3 (PR3) and myeloperoxidase (MPO) levels by classical and automated multiplex microbead assay testing. PR3- and MPO-ANCA were determined by enzyme-linked immunosorbent assay (ELISA) and multiplex CytoBead microbead assay (MIA) in 118 patients with ANCA-associated vasculitis as disease criterion and in 300 patients with autoimmune and gastrointestinal diseases, 49 with infectious disorders, and 125 blood donors (BD) as control criterion. MPO-ANCA detected by MIA demonstrated a significantly higher AUC compared with those determined by ELISA. AUC, area under the curve; CI, confidence interval.
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Table 4. Comparison of classical with automated anti-neutrophil cytoplasmic antibody (ANCA) endpoint-titer analysis.

Classical (1/titer)										Automated (1/titer)	
	= <10	20	40	80	160	320	640	1280	2560	5120	N
= <10	402 (99.3%)	2 (0.5%)	1 (0.2%)	0	0	0	0	0	0	0	405
20	8 (12.3%)	55 (84.6%)	1 (1.5%)	1 (1.5%)	0	0	0	0	0	0	65
40	0	6 (27.3%)	13 (59.1%)	3 (13.6%)	0	0	0	0	0	0	22
80	0	0	5 (26.3%)	12 (63.2%)	2 (10.5%)	0	0	0	0	0	19
160	0	0	0	4 (33.3%)	6 (50.0%)	1 (8.3%)	0	1 (8.3%)	0	0	12
320	0	0	0	0	6 (24.0%)	12 (48.0%)	7 (28.0%)	0	0	0	25
640	0	0	0	0	2 (8.3%)	6 (25.0%)	11 (45.8%)	5 (20.8%)	0	0	24
1280	0	0	0	0	0	1 (7.7%)	4 (30.8%)	7 (53.8%)	1 (7.7%)	0	13
2560	0	0	0	0	0	0	0	0	1 (50.0%)	1 (50.0%)	2
5120	0	0	0	0	0	0	0	0	2 (40.0%)	3 (60.0%)	5
	410	63	20	20	16	20	22	13	4	4	592

ANCA endpoint titers were determined by serial dilution of the 592 samples included in the study by classical indirect immunofluorescence (IIF) and compared to those detected by automated CytoBead IIF on the digital IIF interpretation system Aklides using a 1 to 20 serum dilution only.

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[1,7,13]. However, so called obligatory second-line testing to confirm ANCA reactivity by molecular solid-phase immunoassays have been recommended for various reasons [32]. Indeed, IIF is the simplest multiparametric test available allowing the contemporary sensitive detection of C- and P-ANCA. However, its specificity for GPA is obviously lower than that of the antigen-specific PR3-ANCA assays. Thus, the combination of both IIF and antigen-specific assays was confirmed in several studies to be the optimal strategy for ANCA detection [33].

Currently, such tests such as ELISAs, microbead-based or line immunoassays are well established [13,34,35]. Their over the past years continuously improved performance, particularly regarding the analysis of PR3-ANCA, have questioned the usefulness of IIF for ANCA testing [24,25,36]. Indeed, the need to run two different assay techniques in the recommended two-tier algorithm increases the workload in an already limited in capacity autoimmune laboratory. However, IIF seems to be an indispensable technique in autoimmune diagnostics due to its unsurpassed sensitivity [37]. Apart from ANCA testing, this has been also decisively demonstrated for the assessment of antinuclear antibodies as confirmed by other groups [38,39].

Hence, combination of the advantages of IIF regarding cell-based assays and its potential for multiplexing by microbead immunoassay within on reaction environment could revolutionize autoimmune diagnostics [15,40]. Indeed, combining screening and confirmatory testing for disease-specific autoantibodies will generate many benefits ranging from shorter hands-on times, better reproducibility of results to more cost-effectiveness in particular for larger series of samples due the opportunity of using automation and modern data management. We and others could already proved the usefulness of automated ethN-based ANCA testing employing digital immunofluorescence and pattern recognition on novel automated IIF interpretation systems such as Aklides [26,41–44]. Furthermore, we have shown the usefulness of this new IIF technique for multiplexing analysis of autoantibodies [14,15,45]. Therefore, we attempted to combine both approaches for effective ANCA testing by IIF in one test environment and to develop additionally an automated interpretation method for the simultaneous pattern interpretation of P- as well as C-ANCA on the one hand and quantitative assessment of PR3- and MPO-ANCA on the other. An earlier attempt by the so called mosaic technique employing several tissue- and cell-based assay sets in one reaction environment did not provide quantitative ANCA interpretation [16].

We could demonstrate a very good to good agreement for P- and C-ANCA as well as PR3- and MPO-ANCA testing by classical and novel multiplex CytoBead analysis. As a matter of fact, the prevalences of positive ANCA confirmed by PR3- or MPO-ANCA showed a very good agreement for both methods. To the best of our knowledge, this is the first report of a combined quantitative screening and confirmatory testing for the serology of ANCA-associated vasculitis. The use of a lower dilution (1 to 20) for PR3- and MPO-ANCA analysis by microbead immunoassay within the reaction environment of the CytoBead technology did not result in a poorer assay performance compared with even third-generation assays for PR3-ANCA. The lower dilution seems to provide a better reaction environment resulting in higher sensitivity mainly for MPO-ANCA. We detected a significantly higher MPO-ANCA prevalence in patients with GPA by the microbead immunoassay compared with ELISA. However, this elevated prevalence of MPO-ANCA could be due to false positive results and needs to be confirmed by further studies. Furthermore, the CytoBead analysis has a greater dynamic range by employing

fluorescence instead of optical density measurement in ELISA subjected to the Lambert–Beer law.

The combination of IIF and autoantigen-specific microbead immunoassay resulted in an improvement of the specificity of ANCA testing. In particular patients with RA and AIH type 1 demonstrated a high prevalence of ANCA on ethN not confirmed by molecular PR3- or MPO-ANCA analysis. This phenomenon could be observed for classical as well as multiplex testing in this study and classical ANCA particularly atypical ANCA on ethN have been found in various other diseases than ANCA-associated vasculitis [11,46–48].

The different median ages of the patient cohorts could have an influence on the ANCA assessment. Elevated titers of antinuclear antibodies have been reported in aging individuals leading to a lower specificity of antinuclear antibody testing regarding this population. Since antinuclear antibody positivity could lead to false positive P-ANCA results, a lower false positive rate could be expected in the control groups with lower median ages such as AIH 1, AIH 2, CD, PSC, and UC. However, this was not the case in this study except for patients suffering from UC and PSC regarding PR3-ANCA in particular. Furthermore, our data confirmed recent data of PR3-ANCA positive patients suffering from UC and PSC detected by sensitive assay techniques [49,50]. Thus, PR3-ANCA might be even proposed as diagnostic parameter for these clinical entities [51]. However, the majority of positive ANCA detected by ethN-based IIF employing both classical and automated IIF were not confirmed by PR3- or MPO-ANCA in these patient cohorts hinting to the presence of other neutrophilic autoantigenic targets.

Quantitative PR3- and MPO-ANCA analysis by multiplex CytoBead technology was at least equal or better compared to classical ELISA testing according to ROC curve analysis. Furthermore, automated endpoint ANCA titer analysis by only one serum dilution using automated IIF interpretation demonstrated a very good agreement with the classical one. Damoiseaux and colleagues could also demonstrate efficient endpoint ANCA titer analysis without serial dilution of samples using digital IIF [51,52]. We and others have shown the usefulness of automated endpoint titer analysis for other autoantibodies such as ANA [20,21,43,44]. Thus, automated IIF combining screening and confirmatory ANCA analysis simultaneously in one reaction environment appears to be a unique opportunity to replace the time-consuming classical two-tier ANCA testing by a one-step analysis. This is especially important for the emergency diagnostics of rapid progressive glomerulonephritis as an oligosymptomatic manifestation of ANCA-associated vasculitis.

Conclusions

The CytoBead technology combining screening and confirmatory PR3- and MPO-ANCA testing simultaneously is an alternative to the conventional two-tier ANCA analysis algorithm, which comprises the screening on ethN and confirmation with molecular solid-phase immunoassays. It can be employed for the sensitive and specific detection of ANCA in patients with ANCA-associated vasculitides and probably in patients with UC and PSC as shown elsewhere previously.

The use of digital IIF interpretation systems provides the opportunity to perform automated and standardized quantitative ANCA testing which meets with the demand of modern autoimmune diagnostics in particular for emergency ANCA analysis.

The novel CytoBead technology enables the simultaneous detection of autoantibodies by cell- and microbead-based immunoassays in one reaction environment and, thus, represents an ideal platform for multiplexing of other autoimmune disease-specific antibodies.

Author Contributions

Performed the experiments: MS IK KG NR RH. Analyzed the data: KC D. Reinhold UA EC PS D. Roggenbuck. Contributed reagents/materials/analysis tools: KC PLM MOB DPB EC. Contributed to the writing of the manuscript: MS D. Roggenbuck. Developed the CytoBead ANCA assay: MS KG. Carried out indirect immunofluorescence assays: MS KG NR. Developed the pattern recognition algorithms: RH.

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Der CytoBead-Assay – Eine neue Möglichkeit der multiparametrischen Autoantikörperanalytik bei systemischen Autoimmunerkrankungen

The CytoBead assay – a novel kind of multiparametric autoantibody analysis in the diagnostics of systemic autoimmune diseases

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Zusammenfassung: Bei Verdacht auf Vorliegen einer systemischen Autoimmunerkrankung wird für die serologische Routinediagnostik ein Zwei-Stufen-Verfahren empfohlen. Zuerst werden Autoantikörpern (AAK) mittels sensitiver zellbasierter indirekter Immunfluoreszenz (IIF)-Teste bestimmt. Ein positives Ergebnis muss aufgrund der Möglichkeit von falsch-positiven Ergebnissen mit einem weiteren, spezifischen Test bestätigt werden. Dieses sukzessive Vorgehen ist notwendig, da zurzeit keine Assaytechnik die notwendigen Anforderungen an ein einstufiges Verfahren hinsichtlich Sensitivität und Spezifität erfüllt. Im Sinne einer effektiven AAK-Diagnostik kann heute schon eine simultane Bestimmung von mehreren AAK mittels multiparametrischer Bestätigungstests die Diagnosefindung im Vergleich zu konventionellen, monoparametrischen Tests wesentlich verkürzen. Jedoch erlauben die verfügbaren multiparametrischen AAK-Nachweismethoden nicht die Kombination von Screening- und Bestätigungstesten. Deshalb wurde basierend auf der digitalen Fluoreszenz mit der hier vorgestellten CytoBead Technologie ein neuer Ansatz entwickelt. Ziel war die Kombination der empfohlenen Stufendiagnostik bestehend aus sensitivem Screening und spezifischer Bestätigungsdiagnostik in einer Reaktionsumgebung und darüber hinaus die Möglichkeit der Adaption auf die serologische Diagnostik mehrerer Autoimmunerkrankungen. Durch a) die Nutzung von Standardglasobjektträgern, b) die Kombination von nativen zellulären oder Gewebesubstraten mit antigenbeladenen fluoreszierenden Mikropartikeln (Beads) in einer Reaktionsumgebung, c) die Möglichkeit der manuellen und automatischen Auswertung mittels IIF und d) die Erhebung von quantitativen Fluoreszenzmessergebnissen konnten die Nachteile der bisher bestehenden Testsysteme überwunden werden. Das neue Prinzip ist auf verschiedene multiparametrische AAK-Nachweise wie zum Beispiel die Bestimmung von antinukleären Antikörpern und AAK gegen entsprechende nukleäre und zytoplasmatische autoantigene Zielstrukturen anwendbar. Damit wurde weiterhin die Basis für die simultane AAK-Multiparameterbestimmung für die Serologie der Zöliakie und von ANCA-assoziierten systemischen Vaskulitiden geschaffen.

Schlüsselwörter: Autoantikörper; Bestätigungsdiagnostik; indirekte Immunfluoreszenz; Mikropartikel; Multiparameterdiagnostik; Screening.

Abstract: If there is a suspicion of a systemic autoimmune disease, a two-step assessment of autoantibodies (AAb) is

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recommended for the serological diagnosis routine. First, AAb will be determined using sensitive, cell-based indirect immunofluorescence. Then, a positive result must be confirmed with a more specific test due to the possibility of false-positive results. This gradual approach is necessary because there is currently no assay technique that fulfills the requirements for a one-stage procedure for sensitivity and specificity. For effective AAb analysis, simultaneous determination of several AAb with multiparametric confirmatory assays significantly shortens serological diagnosis, compared with conventional monoparametric testing. Yet, currently available multiparametric AAb detection techniques do not offer the combination of screening and confirmatory testing. Thus, a new approach based on digital fluorescence was developed by applying a novel CytoBead technology that is presented here. The aim was to combine the recommended stepwise approach consisting of sensitive screening and confirmation of specific diagnosis in a reaction environment and beyond the possibility of adaptation to the serological diagnosis of several autoimmune diseases. Using standard microscopic glass slides and the combination of native cellular or tissue substrates with autoantigen-loaded fluorescent microparticles (beads) in a reaction environment, along with the possibility of manual and automatic evaluation by IIF and the quantitative measurement of fluorescent signals, the disadvantages of currently existing test systems could be overcome. This novel concept is applicable for the determination of various multiparametric AAb, e.g., the determination of antinuclear antibodies and the corresponding AAb in molecular cytoplasmic and nuclear autoantigenic structures. Further, this becomes the basis for the simultaneous multiparametric AAb determination for the serology of celiac disease or ANCA-associated vasculitides.

Keywords: Autoantibody; confirmation testing; indirect immunofluorescence; microparticle; multiparameter diagnostic; screening.

Einleitung

Die Labordiagnostik von systemischen Autoimmunerkrankungen (SAIE) umfasst die Bestimmung von Entzündungsparametern sowie erkrankungsspezifischen Autoantikörpern (AAK) [1–5]. Während erstgenannte Parameter auf entzündliche Prozesse unabhängig von deren Ursachen hinweisen, können erkrankungsspezifische AAK als Zeichen einer autoimmunen Pathogenese gewertet werden. AAK können daher richtungsweisend für die Diagnostik und Therapie von SAIE sein [3, 4, 6]. Bei der Mehrzahl der bekannten SAIE sind mehrere AAK mit diagnostischer und/oder prognostischer Relevanz nachweisbar und deren Bestimmung hat Eingang in die Klassifikationskriterien solcher SAIE gefunden [7–9]. Aus Gründen der Kosten- und Zeitersparnis wird heute immer mehr diskutiert, alle für die Erkrankung relevanten AAK in einem Testansatz mittels multiparametrischer Teste zu bestimmen [10–14].

Bedeutung der multiparametrischen Biomarkeranalytik bei Autoimmunerkrankungen

Aufgrund der großen Variabilität in den klinischen Manifestationen und dem meist langen präklinischen Stadium von SAIE kommt der Biomarkeranalytik eine besondere Bedeutung zu. Die klinische Diagnostik von SAIE wird meist durch einen unspezifischen und variablen Beginn der Erkrankung erschwert. Eine frühzeitige Bestimmung erkrankungsspezifischer AAK (z.B. CCP-AAK bei Verdacht auf rheumatoide Arthritis) kann richtungsweisend für die weitere Diagnostik und Therapie sein (Abbildung 1) [4, 15, 16].

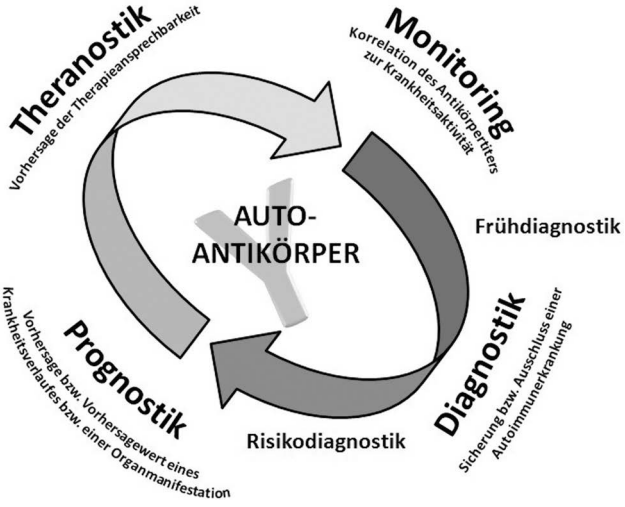

Abbildung 1 Schematische Darstellung klinischen Relevanz von Autoantikörpern.
Erkrankungsspezifische Autoantikörper sind richtungweisend in der (Früh)Diagnostik der assoziierten Autoimmunerkrankung. Darüber hinaus können sie wertvolle Hinweise bezüglich Erkrankungsentwicklung, Therapieansprechen und Krankheitsaktivität liefern.

Eine Vielzahl der diagnostisch relevanten AAK ist bereits präklinisch nachweisbar [15–18]. Bei simultaner Bestimmung von mehreren AAK mittels multiparametrischer Assays kann die Zeit der Diagnosefindung im Vergleich zur konventionellen Stufendiagnostik verkürzt werden [19, 20]. Mit Zunahme an nachzuweisenden Parametern steigt die Wahrscheinlichkeit, einen möglichen Verdacht auf eine SAIE zu bestätigen bzw. auszuschließen [1, 21].

Neben der Zeiteinsparung sind auch Automatisierbarkeit und (in Abhängigkeit von der technologischen Lösung) die deutliche Kostenreduktion entscheidende Argumente, die für den Einsatz multiparametrischer Teste sprechen. Darüber hinaus ergeben sich zahlreiche Vorteile aus klinisch-diagnostischer Sicht. Mit Zunahme der nachzuweisenden Parameter in einem Assay steigt die Sicherheit der Entscheidung bei Verdacht einer SAIE. Die Antigendiversität eines multiparametrischen Assays kann auf alle diagnostischen Fragestellungen angepasst werden und somit u.a. auch eine zuverlässigere Identifizierung von bestimmten Überlappungssyndromen ermöglichen.

Das Prinzip: Screening und Bestätigung

Der gegenwärtige Standard in der Routinediagnostik bei Verdacht auf Vorliegen einer SAIE (insbesondere einer ANA-assoziierten rheumatischen Erkrankung, AARE) ist die Kombination eines hoch sensitiven Screeningtestes mit nachfolgender Bestimmung der spezifischen Marker-Antikörper [22]. AARE, auch als Kollagenosen bezeichnet, umfassen den systemischen Lupus erythematodes (SLE), die systemische Sklerose (SSc), das Sjögren-Syndrom (SjS), autoimmune Myositiden (AIM) sowie verschiedene Mischkollagenosen (z.B. Sharp-Syndrom). Diese Systemerkrankungen sind charakterisiert durch die Produktion zahlreicher nicht-organspezifischer, vorwiegend antinukleärer (ANA) aber auch antizytoplasmatischer Antikörper, welche mit Ausnahme einiger Myositis-spezifischer AAK mittels Immunfluoreszenzscreening an HEp-2-Zellen erfasst werden [23–27]. In Abhängigkeit von klinischer Fragestellung und Fluoreszenzmuster an HEp-2-Zellen erfolgt danach die Bestimmung der entsprechend relevanten AAK mittels spezifischer Immunoassays. Dieses sukzessive Vorgehen hat gegenüber der solitären Testung von krankheitsassoziierten AAK-Spezifitäten mehrere Vorteile [1, 28]: (a) Bei Negativität im Screeningtest kann eine Reihe von AARE (v.a. SLE und Sharp-Syndrom) relativ sicher ausgeschlossen werden. (b) Der HEp-2-Zell-Assay ermöglicht ein multiparametrisches hoch sensitives Screening auf mehr als 30 klinisch relevante AAK-Spezifitäten und damit eine Steigerung der Sensitivität der Diagnostik bei AARE mit unabhängig exprimierten Marker-Antikörpern (z.B. SSc). (c) Die Musterdifferenzierung erlaubt neben einer spezifischen Diagnostik (z.B. Anti-Zentromer-Antikörper) zahlreiche Hinweise auf zu Grunde liegende klinisch relevante AAK-Spezifitäten wie dsDNA- und DFS70-Antikörper [29]. (d) Es können klinisch relevante zusätzliche oder gar Zufallsbefunde (z.B. anti-mitochondriale Antikörper bei primär biliärer Zirrhose mit initialen rheumatischen Beschwerden) erhoben werden. (e) Die Screeningbefunde geben Hinweise auf mögliche falsch-positive Ergebnisse in den spezifischen Immunoassays (z.B. positive dsDNA-Antikörper bei negativen ANA) und erhöhen damit die diagnostische Sicherheit.

Die Qualität der Diagnostik wird also durch die Kombination von hoch sensitiven Screening- mit hoch spezifischen Bestätigungstesten gesteigert [30, 31]. Ein hoch sensitiver Screeningassay erfüllt im Wesentlichen die Rolle einer Ausschlussdiagnostik aufgrund seines hohen negativen prädiktiven Wertes [32, 33]. Ein positives Ergebnis gibt andererseits einen wichtigen Hinweis, beweist aber weder das Vorliegen, noch erlaubt es die sichere Diagnose einer Autoimmunerkrankung. Das Screening erfüllt die Bedeutung als Suchtest nach AAK, bei dem ein gewisser Anteil an falsch-positiven Resultaten akzeptiert wird [34]. Bei einem positiven Testergebnis mittels Screeningassay erfolgt die Bestätigung der Verdachtsdiagnose mit einem spezifischem Immunoassay. Der Bestätigungstest besitzt eine wesentlich höhere diagnostische Spezifität und einen höheren positiven prädiktiven Wert als der Suchtest, schließt jedoch falsch positive Befunde nicht aus [35, 36]. In der Notfalldiagnostik bei Nierenmanifestation ANCA-assoziierter Vaskulitiden ist daher im Interesse einer höchstmöglichen diagnostischen Sicherheit das Screening auf C-/P-ANCA mittels IIF an neutrophilen Granulozyten parallel mit der spezifischen Bestimmung von Myeloperoxidase (MPO)- und Proteinase 3 (PR3)-ANCA gefordert [1, 4, 33].

Multiparametrische Nachweismethoden für Autoantikörper

Heute sind zahlreiche multiparametrische Nachweismethoden für Autoantikörper verfügbar, die sich vor allem durch Testmatrix und Messmethode unterscheiden (Tabelle 1). Das Grundprinzip basiert auf immobilisierten Biomolekülen, die über verschiedene Verfahren detektiert werden.

Tabelle 1 Übersicht multiparametrischer Nachweismethoden für Autoantikörper.

Methode	Chemolumi-neszenz	CytoBead	Einzel-ELISA	Mosaik-Biochip	Screen-ELISA	SeraSpot	Streifentest	Zelluläre Assays
Firma	Inova (Bioflash) Menarini (Zeuss)	Medipan (Aklides)	verschiedene	EuroImmun	verschiedene	Seramun	verschiedene	verschiedene
Prinzip	Protein beladene Beads	Monolayer aus Zellen/ Gewebe, kombiniert mit Protein beladenen Beads	einzelne Proteine kombiniert in Multiwellplatten	Monolayer aus Zellen/ Gewebe kombiniert mit Proteinspots	Proteinmix in Multiwellplatte	Proteinspots in Multiwellplatte	Proteinspots auf Membran	Monolayer aus Zellen oder Gewebe
Nachweis	Bestätigung	Screening (Zellen/ Gewebe), Bestätigung (Beads)	Bestätigung	Screening (Zellen), Bestätigung (Proteinspots)	Screening	Bestätigung	Bestätigung	Screening
Methode	sequentiell	parallel	sequentiell	parallel	parallel	parallel	parallel	parallel
Messsystem	Hersteller-spezifisch	manuelles Mikroskop, Aklides	ELISA-Reader	manuelles Mikroskop	ELISA-Reader	Hersteller-spezifisch	Scanner	manuelles Mikroskop
Auswertung	automatisch	manuell, automatisch	automatisch	manuell	automatisch	automatisch	manuell, automatisch	manuell automatisch
Ergebnis	quantitativ	semi-quantitativ (Zellen/Gewebe), quantitativ (Beads)	quantitativ	semi-quantitativ	semi-quantitativ	semi-quantitativ	semi-quantitativ	semi-quantitativ
Multiplexgrad	1	variabel	1	variabel, meist <12	hoch	bis 24	variabel, bis 24	sehr hoch (z. B. HEp-2 Zelle: >3000)
Zeitbedarf	~30 min	~1,5 h	variabel, ~2 h	variabel, ~1,5 h	variabel, meist 1–2 h	~2 h	variabel, meist 1–2 h	variabel, ~1,5 h
Literatur	Pelkum et al. 2014 [31]	Sowa et al. 2014 [11]		Sayegh et al. 2014 [37]				Hiemann et al. 2009 [28]

Hinweis: Real quantitative Messergebnisse sind ausschließlich bei Mitführung von Kalibratoren für jeden Parameter möglich.

Testmatrix

Eine Testmatrix ist die Unterlage und das Format, auf dem Proteine oder Peptide immobilisiert werden. Am weitesten verbreitet sind Proteinauftragungen in Form von Spots oder Linien auf Membranen – sogenannte Streifenteste. Weiterhin können sowohl Multiwellplatten als auch Glasobjektträger mit Proteinen beschichtet werden. Die Vorteile dieser proteinbeladenen Matrizen liegen in der einfachen Handhabung und sehr günstigen manuellen oder automatischen Auswertung über angeschlossene Scanner mit Auswertesoftware. Nachteilig sind Messgenauigkeit aufgrund fehlender Kalibratoren (semi-quantitativ) und niedrige Sensitivität wegen densitometrischer Bestimmung einer Farbumschlagsreaktion. Letzteres konnte von einzelnen Herstellern durch Fluoreszenzmarkierung der Detektormoleküle und der damit verbundenen Lichtquantendetektion verbessert werden.

Alternativ werden Mikropartikel (Beads) aus Polystyrol (PS) oder Polymethylmethacrylat (PMMA) mit einem Durchmesser bis 20 µm als feste Phase für die Entwicklung der Reaktionsumgebung eingesetzt [13, 14, 19]. Einpolymerisierte Fluoreszenzfarbstoffe sowie verschiedene Beadgrößen erlauben die Unterscheidung einzelner Populationen. Auf der Oberfläche der Beads sind hochgereinigte Autoantigene in natürlicher oder rekombinanter Form immobilisiert. Diese ermöglichen eine spezifische Detektion von AAK. Ein Vorteil der Beads ist, dass einzelne Populationen individuell oberflächenmodifiziert werden können, was spezifische Immobilisierungsstrategien für Biomoleküle generiert. Daraus ergeben sich Möglichkeiten der Reaktionsumgebungsanpassung hinsichtlich Proteinfaltung, aber auch der gezielten Beeinflussung von anderen Leistungsparametern. Aufgrund der geringen Größen sind viele Beads als individuelle Messpunkte kombinierbar und garantieren bei der Messwertberechnung robuste statistische Verteilungen. Die Messung der Fluoreszenz oder auch Chemolumineszenz ist sehr sensitiv und mitgeführte Kalibratoren ermöglichen reale quantitative Messungen über chargenspezifische Kalibrierkurven.

Von Nachteil sind herstellerspezifische Messsysteme, die eine Messung von Testen anderer Anbieter meist nicht ermöglichen. Manuelle Analysen der Bead-basierten Assays waren bislang nicht möglich.

Messsysteme

Messsysteme für Antikörpernachweise sind Scanner für Farbumschlagsreaktionen auf Streifentesten, Durchflusszytometer für Beads sowie Fluoreszenzmikroskope für Proteinspots und Beads.

Scannersysteme sind einfach in der Handhabung und kostengünstig, nachteilig ist jedoch der Charakter der Dokumentation für Streifenteste. Semi-quantitative Messungen und Analysen sind möglich, die hohe Präzision von Fluoreszenz- oder Chemolumineszenz-basierten Systemen können diese Systeme aufgrund der densitometrischen Auswertung allerdings nicht erreichen.

Zytometer detektieren und messen Beads hinsichtlich Größe und Fluoreszenzintensität im Durchfluss und ermöglichen präzise quantitative Messungen. Die Messung erfolgt in Standardgefäßen oder speziellen herstellerabhängigen Kartuschen sequentiell als Einzelbeads oder als parallele Multiplexmessung der Beadmischung. Ausgewertet wird die Fluoreszenz an der Beadoberfläche, deren Intensität mit der AAK-Konzentration korreliert. Nachteilig sind die hohen Anschaffungskosten für das Messsystem sowie die einmalige Beadmessung, die nicht wiederholt werden kann. Die Möglichkeit, komplexere Autoantigensubstratsysteme z.B. Gewebeschnitte für die AAK-Bestimmung zu verwenden, kann durch das Durchflusszytometer bisher nicht realisiert werden.

Mikroskope, welche manuell oder automatisiert über entsprechende Software gesteuert sind, werden für die Analyse von fluoreszenzbasierten Assays verwendet. Beads und Proteinspots werden auf planaren Oberflächen in Standardformaten wie Multiwellplatten und Glasobjektträger analysiert. Neben artifiziellen Substraten wie antigenbeschichtete Beads sind auch native Substrate wie Zellen und Gewebe, die beim Autoantikörperscreening eingesetzt werden, detektier- und messbar [28, 38]. Die weite Verbreitung der Fluoreszenzmikroskope und die herstellerunabhängigen flexiblen Einsatzmöglichkeiten sind die Hauptvorteile. Quantitative Messungen und die Kontrolle der Probe durch Wiederholungsmessungen sind möglich.

Alle aktuell verfügbaren multiparametrischen Nachweismethoden für den Autoantikörpernachweis erlauben jedoch aufgrund der Beschränkung auf ein Nachweissystem nicht die kombinierte Analytik von Screening- und Bestätigungstest.

Das CytoBead-Prinzip

Basierend auf den Erfahrungen mit bestehenden Verfahren hinsichtlich der genannten Vor- und Nachteile wurde beim CytoBead-Prinzip eine Weiterentwicklung

angestrebt. Ziel der Entwicklung war ein einfaches Nachweissystem, welches die Stufendiagnostik von AAK mit sensitivem Screening und spezifischer Bestätigungsdiagnostik kombiniert und darüber hinaus auf verschiedene Autoantikörpernachweise adaptierbar macht. Durch Kombination innovativer neuer Ansätze, die im Folgenden beschrieben werden, konnten die Nachteile bestehender Testsysteme überwunden werden.

Multiple Auftragsstellenkompartimente

Die Stufendiagnostik erfordert den Nachweis der AAK auf unterschiedlichen autoantigenen Substraten. Um eine Kombination dieser Testsysteme zu ermöglichen, wurden einzelne Auftragsstellen auf konventionellen Glasobjektträgern durch Barrieren aus Teflon in Teilkompartimente unterteilt (Abbildung 2A). Somit entstehen Testsysteme für erkrankungsspezifische Kombinationen, die spezifische Nachweissysteme für die Profildiagnostik ermöglichen. Für die Abarbeitung dieses Tests ergeben sich im Vergleich zur konventionellen IIF, z.B. in der ANA- und ANCA-Diagnostik, keine Unterschiede [11]. Die Fluidik des Serum- und Konjugattropfens verhält sich äquivalent zum Standard-Glasobjektträger mit klassischen Auftragsstellen. Der regelmäßige Abstand im 96-Kavitätenraster ermöglicht die manuelle und automatische Testabarbeitung.

Zellen, Gewebe + Beads

Die Unterteilung in multiple Auftragsstellenkompartimente ermöglicht die Kombination von verschiedenen Methoden. HEp-2 Zellen werden z.B. beim Nachweis von ANA als sensitives Screeningsystem mit einem Repertoire von mehr als 30 klinisch relevanten Autoantigenen eingesetzt. Darüber hinaus können Granulozyten als Substrat für den ANCA-Nachweis und *Crithidia luciliae* als spezifischer anti-dsDNA-AAK Test genutzt werden [11, 20, 39, 40]. Für den Nachweis von organspezifischen AAK werden Gewebeschnitte von Ratten oder Affen aus Speiseröhre, Leber, Magen oder Niere verwendet als auch rekombinant in Zellen exprimierte Autoantigene [41, 42].

Durch angepasste Oberflächenmodifikationen können im mittleren Kompartiment Zellen oder Gewebeschnitte immobilisiert werden und bilden mit Beads in den äußeren Kompartimenten ein Testprofil. Auf einem Testsystem ist somit sensitives Screening auf nativen Strukturen und spezifische Bestätigung mit Hilfe einer Festphase, den proteinbeladenen Beads, möglich (Abbildung 2B).

Manuelle und automatische Auswertung

Neue Messmethoden sind meist eng gekoppelt an neue Auswertesysteme, die ein Auslesen der Messdaten

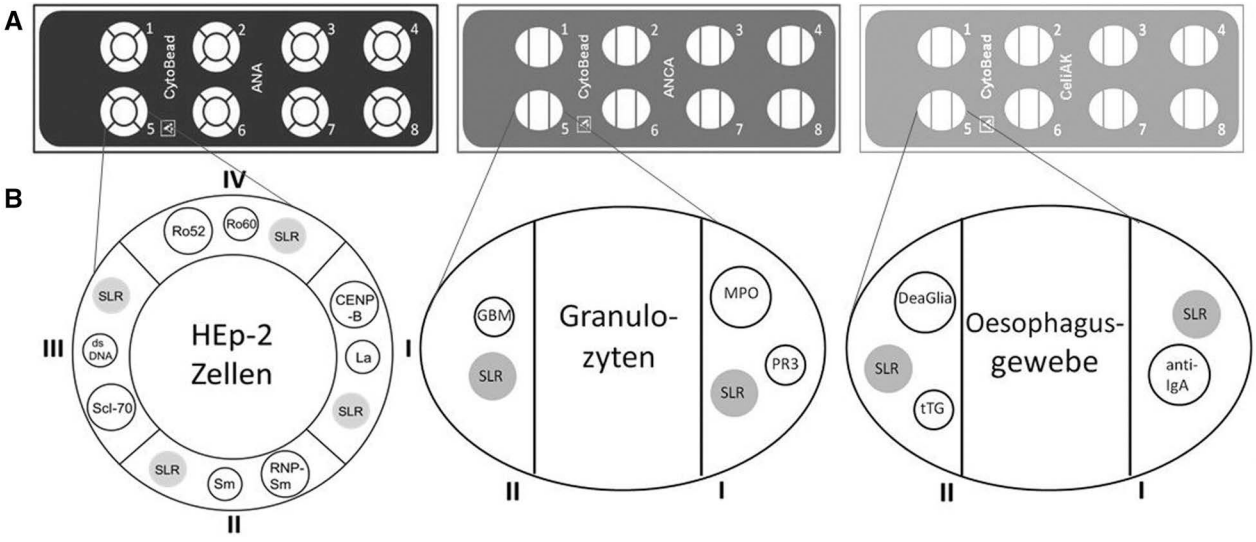

Abbildung 2 CytoBead Objektträgerausführungen mit 8 Auftragsstellen für unterschiedliche Testprofile.
Kombination aus Screeningtest mit nativem Substrat der Zellen oder Gewebe (Zentrumskompartiment) und artifiziellem Substrat durch Antigen-beladene fluoreszierende Mikropartikel (periphere Kompartimente). CytoBead ANA (links), ANCA (Mitte) und Zöliakie (rechts). SLR ist die Bezeichnung der Referenzbeads für die manuelle Beadklassifikation.

ermöglichen. Hohe Investitionskosten und mangelnder Laborplatz verhindern teilweise die Nutzung neuer Methoden. In fast jedem diagnostischen Autoimmun-Routinelabor ist ein Fluoreszenzmikroskop mit grünem Filter für Fluoresceinisothiocyanat (FITC) verfügbar und wird traditionell zur manuellen, klassischen Analyse der in den 70er Jahren entwickelten IIF-Teste angewendet.

Das neu etablierte Objektträgerformat in Kombination mit einer grünen Fluoreszenz im FITC-Wellenlängenbereich für die Signaldetektion ermöglicht erstmalig die Auswertung von Beadreaktivitäten an konventionellen manuellen Routinemikroskopen. Für diese qualitative bis semi-quantitative Auswertung per Auge ist kein spezielleres Messsystem notwendig. Die Größeneinteilung der rotfluoreszierenden, antigenbeladenen Beads in verschiedene Populationen für die manuelle Bewertung wird durch Referenzbeads unterstützt. Diese homogen grünfluoreszierenden Beads dienen als Größenmaßstab und erlauben die sichere manuelle Identifizierung und Zuordnung.

Die in den letzten Jahren entwickelten Interpretationssysteme für die automatische Analyse von IIF Testen können äquivalent für die Analyse der kombinierten CytoBead-Teste verwendet werden [28, 43–46].

Quantifizierung

Internationale Vergleichbarkeit von Messergebnissen erfordert kalibrierte Systeme, die semi-quantitative oder quantitative Messergebnisse ausgeben [47, 48]. Für quantitative Analysen sind für den Ausgleich von eventuellen chargenspezifischen und geräteabhängigen Schwankungen Kalibratoren mitzuführen. Daraus ergibt sich, dass manuell ausgewertete Teste und Teste ohne Kalibratoren bestenfalls semi-quantitative Ergebnisse liefern können. Bei manueller Auswertung der CytoBead-Teste sind semi-quantitative Aussagen vergleichbar mit Aussagen von Streifentesten.

Bei Messungen mit automatischen Systemen wie Aklides (Medipan, Dahlewitz) und der Mitführung von Kalibratoren ist über chargenspezifische Masterkurven eine Ausgabe der Messwerte in internationalen Einheiten (IE/mL) wie bei konventionellen ELISA möglich [11].

Zusammenfassend vereinigt das CytoBead-Prinzip die konventionelle Stufendiagnostik verschiedener Testsysteme in einem Testansatz. Die Auswertung kann sowohl manuell an konventionellen Fluoreszenzmikroskopen als auch an modernen automatisierten Mikroskopen erfolgen. Die automatische Auswertung ermöglicht durch Mitführung von Kalibratoren die Ausgabe von Ergebnissen in internationalen Einheiten. Das Prinzip ist anwendbar auf verschiedene Autoantikörpernachweise für die serologische Diagnostik von Kollagenosen (ANA-Screening plus Bestimmung Kollagenose-assoziierter ANA-Spezifitäten), ANCA-assoziierten Vaskulitiden (ANCA-Screening plus Bestimmung der ANCA-Spezifitäten) aber auch organspezifischen Autoimmunerkrankungen (Abbildung 2).

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The CytoBead assay – a novel approach of multiparametric autoantibody analysis in the diagnostics of systemic autoimmune diseases

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Abstract: If there is a suspicion of a systemic autoimmune disease, a two-step assessment of autoantibodies (AAb) is recommended for the serological diagnosis thereof. First, AAb will be determined using sensitive, cell-based indirect immunofluorescence. Then, a positive result must be confirmed with a more specific test due to the possibility of false-positive results. This gradual approach is necessary because there is currently no assay technique that fulfills the requirements for a one-stage procedure for sensitivity and specificity. For effective AAb analysis, simultaneous determination of several AAb with multiparametric confirmatory assays significantly shortens serological diagnosis, compared with conventional monoparametric testing. Yet, currently available multiparametric AAb detection techniques do not offer the combination of screening and confirmatory testing. Thus, a new approach based on digital fluorescence was developed by applying a novel CytoBead technology that is presented here. The aim was to combine the recommended stepwise approach consisting of sensitive screening and confirmation of specific diagnosis in a reaction environment and thereafter the possibility of adaptation to the serological diagnosis of several autoimmune diseases. Using standard microscopic glass slides and the combination of native cellular or tissue substrates with autoantigen-loaded fluorescent microparticles (beads) in a reaction environment, along with the possibility of manual and automatic evaluation by IIF and the quantitative measurement of fluorescent signals, the disadvantages of currently existing test systems could be overcome. This novel concept is applicable for the determination of various multiparametric AAb, e.g., the determination of antinuclear antibodies and the corresponding AAb in molecular cytoplasmic and nuclear autoantigenic structures. Further, this becomes the basis for the simultaneous multiparametric AAb determination for the serology of celiac disease or ANCA-associated vasculitides.

Keywords: autoantibody; confirmatory testing; indirect immunofluorescence; microparticle; multiparametric diagnostics; screening.

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Introduction

The serological diagnostics of systemic autoimmune diseases (SAD) includes the determination of inflammatory parameters and disease-specific autoantibodies (AAb) [1–5]. While the former parameters point to inflammatory processes irrespective of their causes, disease-specific AAb can be seen as an important sign of the autoimmune pathogenesis. AAb can, therefore, serve as a benchmark for the diagnosis and therapy of SAD [3, 4, 6]. In the majority of known SAD, more than a few AAb with diagnostic and/or prognostic relevance can be detected, and their determination has been included in classification criteria

of corresponding SAD [7–9]. For reasons of cost and time savings, there is an ongoing discussion about determining all disease relevant AAb by using multiparametric test approaches [10–14].

The significance of multiparametric biomarker analysis in autoimmune diseases

Regarding their large variability of clinical manifestations and the mostly long pre-clinical stage of SAD the analysis of biomarkers is of special importance. The clinical diagnosis of SAD is often difficult due to the non-specific and variable onset of the disease. An early identification of disease-specific AAb (e.g., anti-CCP antibodies when rheumatoid arthritis is suspected) may point towards further diagnostics and therapy strategies (Figure 1) [4, 15, 16].

A large number of diagnostic relevant AAb can be detected pre-clinically [15–18]. In contrast to the conventional stepwise diagnostics, multiparametric assays for the simultaneous determination of several AAb in one approach can reduce the time needed to get a medical diagnosis [19, 20]. The higher number of relevant parameters in one assay will correlate with the higher probability of confirmation or exclusion of a specific SAD [1, 21].

Apart from time savings, the automation and (relative to the technological solution) significant reduction of costs are key arguments in favor to use multiparametric assays. In addition, there are numerous advantages from a clinical-diagnostic point of view. The increasing number of parameters which can be detected with one assay also increases the certainty of reaching a decision in the case of suspected SAD. The antigenic diversity of a multiparametric assay can be adapted to all diagnostic investigations, which allows an extensive way a more reliable identification of certain overlapping syndromes.

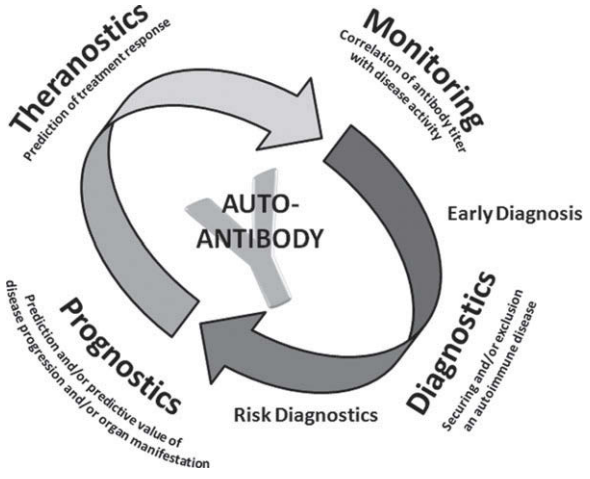

Figure 1: Schematic representation of the guideline for the diagnostics and theranostics of patient's autoantibodies.
The early detection (early diagnosis) of antibodies sets the course for further theranostics – treatment, diagnostics and monitoring of the patient.

The principle: screening and confirmation

The current standard in routine diagnostics where there is a suspicion of SAD (particularly an ANA-associated rheumatic disease, AARD) is the combination of a highly sensitive screening assays followed by specific determination of marker antibodies [22]. AARD, also known as connective tissue diseases (CTD), comprise systemic lupus erythematosus (SLE), systemic sclerosis (SSc), the Sjögren's syndrome (SjS), autoimmune myositis (AIM) and various mixed CTDs (e.g., Sharp syndrome). These systemic diseases are characterized by the production of numerous non-organ-specific, predominantly antinuclear antibodies (ANA) as well as anticytoplasmic antibodies, which, except for a few myositis-specific AAb, can be detected via immunofluorescence screening on HEp-2 cells [23–27]. Depending on the clinical suspicion, a positive fluorescence patterns on HEp-2 cells should be confirmed with antigen-specific immunoassays. This gradual approach has several advantages in contrast to the solitary testing of disease-associated AAb specificities [1, 28]: (a) Negative results of the screening assay can be used to exclude a number of AARD (especially SLE and Sharp syndrome) with high confidence. (b) The HEp-2 cell assay allows highly sensitive multiparametric screening for more than 30 clinically relevant AAb specificities, and, therefore, increases the sensitivity for the diagnosis of AARD with independently expressed marker antibodies in the cell (e.g., SSc). (c) The pattern differentiation, aside from the specific AAb diagnostics (e.g., anti-centromere antibodies), provides diverse indications about underlying clinically relevant AAb specificities, such as dsDNA and DFS70 antibodies [29]. (d) It is possible to obtain clinically relevant or even incidental findings (e.g., anti-mitochondrial antibodies in connection with primary biliary cirrhosis with initial rheumatic symptoms). (e) The screening results distinguish possible false-positive findings in the specific immunoassays (e.g., positive dsDNA antibodies with

negative ANA) and thus increase diagnostic confidence. Thus, the quality of medical diagnostics is improved by the combination of highly sensitive screening and highly specific confirmatory testing [30, 31]. Essentially, a negative result obtained with a highly sensitive screening assay potentially exclude patients under suspicion of AARD due to its high negative predictive value [32, 33]. However, a positive result provides an important indication, but does not prove the presence or allow a reliable diagnosis of an autoimmune disease. Screening is a test method to detect all AAb, where a certain proportion of false-positive findings is accepted [34]. Therefore, a positive test result of a screening assay-has to be confirmed with a specific immunoassay. The confirmation assay has a significantly higher diagnostic specificity and a higher positive predictive value than the screening assay, but does not rule out false-positive findings completely [35, 36]. In the case of emergency situations like ANCA-associated vasculitis involving the kidneys, a maximum diagnostic confidence is needed which requires screening for C/P-ANCA via IIF on neutrophil granulocytes along with the specific determination of myeloperoxidase (MPO)- and proteinase 3 (PR3)-ANCA [1, 4, 33].

Multiparametric methods for detecting autoantibodies

Today, there are many multiparametric methods for detecting autoantibodies, which differ mainly in the test matrix and the measurement method (Table 1). The basic principle is based on immobilized biomolecules which are detected employing different methods.

Test matrix

A test matrix is the substrate and format on which proteins or peptides are immobilized. The most common are applications of proteins in the form of spots or lines on membranes – so-called line dot assays. Furthermore, it is also possible to immobilize proteins to multiwell plates or glass slides. The advantages of these protein-coated matrices are derived from the easy handling and very efficient manual or automated analysis reader systems with evaluation software. However, measurement accuracy due to the lack of calibrators (semi-quantitative) and low sensitivity due to the densitometric determination of a color change reaction are disadvantageous. Various manufacturers managed to improve the latter through the fluorescence labeling of detector molecules and the associated light quantum detection.

Alternatively, microparticles (beads), consisting of polystyrene (PS) or polymethyl methacrylate (PMMA), with a diameter of up to 20 µm are used as solid phase for the development of the reaction environment [13, 14, 19]. Polymerized fluorescence dyes and various sizes of beads allow the differentiation of individual populations. On the surface of the beads, there are immobilized highly purified autoantigens in native or recombinant form. They allow the specific detection of AAb. Furthermore, the possible individual surface modification of each bead population, generates the optimal immobilization strategy for each autoantigen. This creates opportunities for adapting the reaction environment with respect to protein folding, as well as for the targeted modulation of other performance parameters. Given their small sizes, many beads can be combined into individual measuring points and guarantee solid statistical distributions in calculating the measured value. The measurement of fluorescence, or also chemiluminescence, is very sensitive. Additionally, attached calibrators allow real quantitative measurements via lot-specific calibration curves.

A disadvantage in this context are manufacturer-specific proprietary measurement systems. Until now, manual analyses of bead-based assays were not possible.

Measuring systems

Measuring systems for antibody detection include scanners for color change reactions on line dot assays, flow cytometers for beads, as well as fluorescence microscopes for protein spots and beads.

Scanner systems are easy to use and cost efficient but the documentation for line dot assays causes a problem. Semi-quantitative measurements and analyses are possible, but these systems cannot achieve the high precision of fluorescence or chemiluminescence-based systems due to their densitometric evaluation.

Cytometers detect and measure beads in terms of size and fluorescence intensity in the flow and allow accurate quantitative measurements. Measurements are taken in standard vials or special manufacturer-designed cartridges, sequentially as single beads or by parallel multiplex measurements of a bead mixture. The fluorescence on the bead surface is analyzed whereby the fluorescence intensity correlates with the concentration of bound AAb. However, the high acquisition costs for the measurement system and the one-time bead measurement which

Table 1: Overview of multiparametric methods for detecting autoantibodies.

Method	Chemiluminescence	CytoBead	Individual ELISA	Mosaic biochip	Screen ELISA	SeraSpot	Strip test	Cellular assays
Company	Inova (Bioflash) Menarini (Zeuss)	Medipan (Aklides)	Various	Euroimmun	Various	Seramun	Various	Various
Principle	Protein-packed beads	Monolayer of cells/ tissue, combined with protein-loaded beads	Individual proteins combined in multiwell plates	Monolayer of cells/ tissue, combined with protein spots	Protein mix in multiwell plate	Protein spots in multiwell plate	Protein spots on membrane	Monolayer of cells or tissue
Detection	Confirmation	Screening (cells/ tissue), confirmation (beads)	Confirmation	Screening (cells), confirmation (protein spots)	Screening	Confirmation	Confirmation	Screening
Method	Sequential	Parallel	Sequential	Parallel	Parallel	Parallel	Parallel	Parallel
Measuring system	Manufacturer-specific	Manual microscope, Aklides	ELISA reader	Manual microscope	ELISA reader	Manufacturer-specific	Scanner	Manual microscope
Evaluation	Automated	Manual, automated	Automated	Manual	Automated	Automated	Manual, automated	Manual, automated
Result	Quantitative	Semi-quantitative (cells/tissue), quantitative (beads)	Quantitative	Semi-quantitative	Semi-quantitative	Semi-quantitative	Semi-quantitative	Semi-quantitative
Degree of multiplexing	1	Variable	1	Variable, usually <12	High	Up to 24	Variable, up to 24	Very high (e.g., HEp-2 cell: >3000)
Time required	~30 min	~1.5 h	Variable, ~2 h	Variable, ~1.5 h	Variable, usually 1–2 h	~2 h	Variable, usually 1–2 h	Variable, ~1.5 h
Literature	Pelkum et al. 2014 [31]	Sowa et al. 2014 [11]		Sayegh et al. 2014 [37]				Hiemann et al. 2009 [28]

Real quantitative measurement results are possible only when calibrators for each parameter are used.

cannot be repeated are shortcomings. The possibility of using more complex autoantigenic substrates e.g., tissue sections, for the AAb determination has so far not been realized by using flow cytometry.

Microscopes that are either controlled manually or automatically via appropriate software are used for the analysis of fluorescence-based assays. Beads and protein spots are analyzed on planar surfaces in standard formats, such as multiwell plates and glass slides. Apart from artificial substrates, such as antigen-coated beads, native substrates like cells and tissues, which are used in autoantibody screening, can be detected and measured [28, 38]. The wide availability of fluorescence microscopes and their manufacturer independent and flexible uses are the main benefits. Quantitative measurements as well as sample control by repeated measurements are possible.

However, all currently available multiparametric methods for detecting autoantibodies, given their limitation to a single detection system, do not allow the combined analysis of screening and confirmation assays.

The CytoBead principle

Based on experience with existing methods with respect to the advantages and disadvantages mentioned, the CytoBead principle was developed to be a more advanced diagnostic tool. The aim was to generate a simple detection system which combines the stepwise diagnostics of AAb with sensitive screening and specific confirmatory testing that furthermore is adaptable for various autoantibody determinations. By combining innovative and new approaches as described below, it is now possible to overcome the disadvantages of existing test systems.

Multiple parted wells

Stepwise diagnostics requires the detection of AAb on different autoantigenic substrates. To allow these test systems to be combined, wells on conventional glass slides were divided into compartments by Teflon barriers (Figure 2A). This creates test environments for disease-specific combinations allowing specific detection systems for profile diagnostics. Regarding the test performance, there are no differences compared to conventional IIF, as used for ANA and ANCA diagnostics [11]. The fluidics of the serum and conjugate drop is equivalent to the standard glass slide with traditional wells. The regular distance in accordance with the 96-cavity grid enables manual and automated test processing.

Cells, tissues + beads

The creation of multiple compartments makes it possible to combine different methods. Regarding the detection of ANA, HEp-2 cells are used as a sensitive screening system with a repertoire of more than 30 clinically relevant autoantigens. In addition, granulocytes can be used as a substrate for ANCA detection, and *Crithidia luciliae* as a specific target for anti-dsDNA-AAb [11, 20, 39, 40]. For the

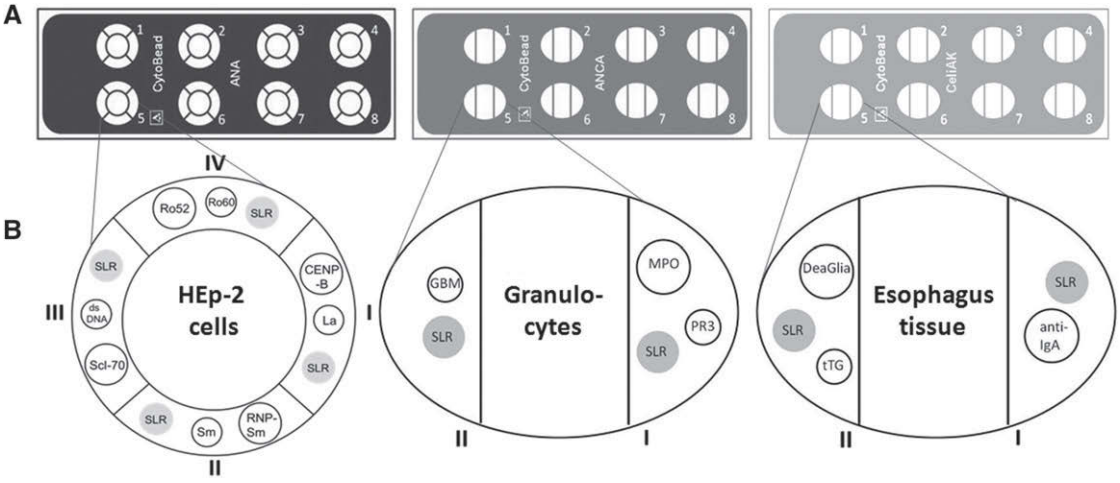

Figure 2: CytoBead slides with eight application points for different test profiles.
Combination of screening with native substrate of cells or tissue (center compartment) and artificial substrate through antigen-loaded fluorescent micro-particles (peripheral compartments). CytoBead ANA (left), ANCA (center) and celiac disease (right). SLR is the designation of reference beads for the manual bead classification.

detection of organ-specific AAb, primate or rat tissue sections from the esophagus, liver, stomach or kidney as well as recombinant autoantigens expressed in specific cell lines are used [41, 42].

Through adapted surface modifications, cells or tissue sections can be immobilized on to the center compartment, which, together with the beads at the outer compartments, create a test profile. As a result, the test system allows sensitive screening on native structures and specific confirmation by solid-phase assay, represented as protein-loaded beads (Figure 2B).

Manual and automated evaluation

New measurement methods are often based on new evaluation systems, which enable the readout of measured data. High investment costs and a lack of laboratory space usually prevent the introduction of new methods. However, almost every diagnostic routine autoimmune laboratory has a fluorescence microscope with a green filter for fluorescein isothiocyanate (FITC), which is used for the manual, traditional analysis of the IIF tests developed in the 1970s.

For the first time, the newly established and unique CytoBead slide format allows the evaluation of bead reactions with conventional manual routine microscopes due to its combination with green fluorescence (FITC wavelength range) for signal detection. This qualitative to semi-quantitative evaluation by eye does not require a special measurement system. The size differentiation of the red fluorescent, antigen-coated beads into different populations for manual evaluation is supported by reference beads. These beads of homogeneous green fluorescence serve as a size scale and enable reliable manual identification and classification.

The interpretation systems developed in recent years for the automated analysis of IIF tests can also be used equally for the analysis of the CytoBead assays [28, 43–46].

Quantification

International comparability of test results requires calibrated systems which yield semi-quantitative or quantitative results [47, 48]. To compensate for any batch-specific and device-dependent fluctuations, it is necessary to use calibrators for quantitative analyses. It follows that manually evaluated tests and tests without calibrators can produce, at best, semi-quantitative results. When evaluating CytoBead tests manually, semi-quantitative statements are comparable to statements obtained from line dot assays.

With measurements involving automated systems like Aklides (Medipan, Dahlewitz) and calibrators, lot-specific master curves can be used to produce values in international units (IU/ml), comparable with conventional ELISA [11].

In summary, the CytoBead principle combines the conventional stepwise diagnostic of different test systems in one single approach. Evaluation can be done manually by using conventional fluorescence microscopes, as well as automatically by modern microscopes. Through the inclusion of calibrators, automated evaluation allows the output of results in international units. The principle can be applied to a variety of autoantibody tests for the serological diagnosis of collagenoses (ANA screening plus determination of collagenose-associated ANA specificities), ANCA-associated vasculitis (ANCA screening plus determination of ANCA specificities), as well as organ-specific autoimmune diseases (Figure 2).

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Next-Generation Autoantibody Testing by Combination of Screening and Confirmation—the CytoBead® Technology

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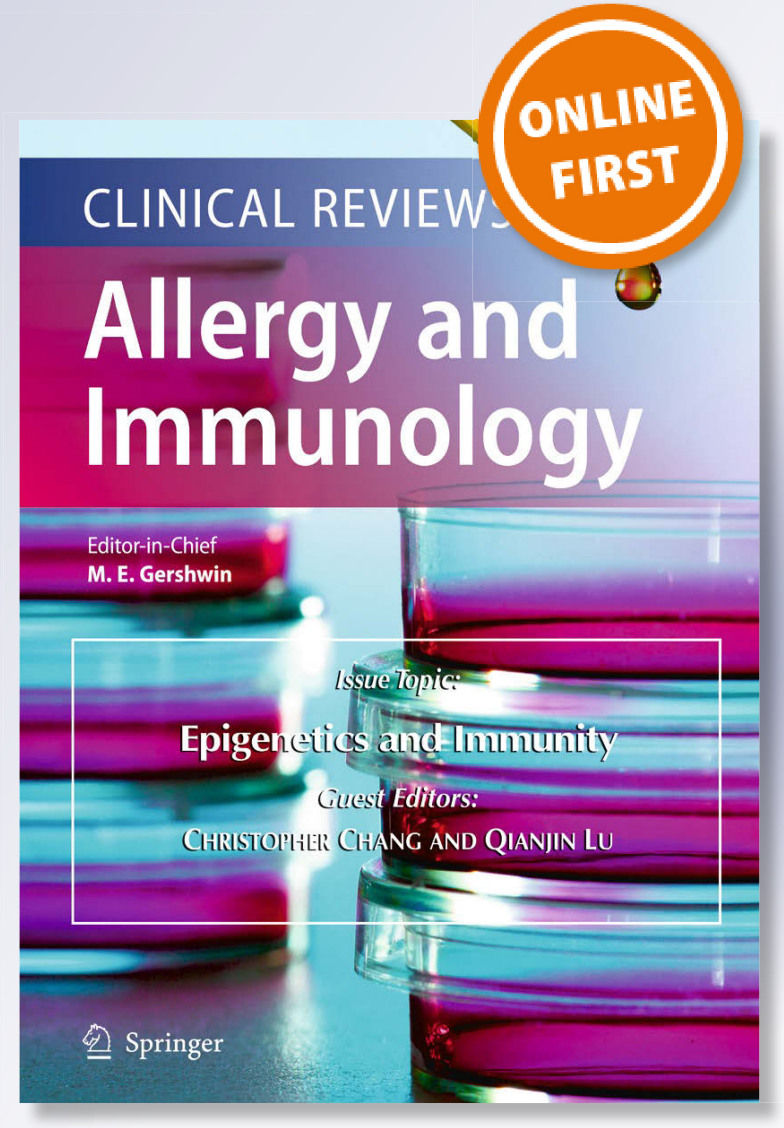

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Next-Generation Autoantibody Testing by Combination of Screening and Confirmation—the CytoBead® Technology

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Abstract Occurrence of autoantibodies (autoAbs) is a hallmark of autoimmune diseases, and the analysis thereof is an essential part in the diagnosis of organ-specific autoimmune and systemic autoimmune rheumatic diseases (SARD), especially connective tissue diseases (CTDs). Due to the appearance of autoAb profiles in SARD patients and the complexity of the corresponding serological diagnosis, different diagnostic strategies have been suggested for appropriate autoAb testing. Thus, evolving assay techniques and the continuous discovery of novel autoantigens have greatly influenced the development of these strategies. Antinuclear antibody (ANA) analysis by indirect immunofluorescence (IIF) on tissue and later cellular substrates was one of the first tests introduced into clinical routine and is still an indispensable tool for CTD serology. Thus, screening for ANA by IIF is recommended to be followed by confirmatory testing of positive findings employing different assay techniques. Given the continuous growth in the demand for autoAb testing, IIF has been challenged as the standard method for ANA and other autoAb analyses due to lacking automation, standardization, modern data management, and human bias in IIF pattern interpretation. To address these limitations of autoAb testing, the CytoBead® technique has been introduced recently which enables automated interpretation of cell-based IIF and quantitative autoAb multiplexing by addressable microbead immunoassays in one reaction environment. Thus, autoAb screening and confirmatory testing can be combined for the first time. The present review discusses the history of autoAb assay techniques in this context and gives an overview and outlook of the recent progress in emerging technologies.

Keywords Second-generation autoantibody testing · Indirect immunofluorescence · Digital fluorescence · Autoimmune disease · Multiplex diagnostics

Autoantibodies as Diagnostic Markers

Connective Tissue Disease-Specific Autoantibodies

The loss of immune tolerance characteristic for connective tissue diseases (CTDs) such as systemic lupus erythematosus (SLE), systemic sclerosis (SSc), poly/dermatomyositis (PM/DM), Sjögren's syndrome (SjS), and mixed connective tissue disease (MCTD) brings about the generation of various nonorgan-specific autoantibodies (autoAbs) [1–3]. Although the triggering factors for the occurrence of autoAbs and their role in the pathogenesis of CTD are still not entirely understood, autoAbs are widely used as diagnostic markers in clinical routine nowadays [4, 5]. The L.E. cell phenomenon described by Hargraves in the late 1940 in patients suffering from SLE proved to be a result of autoAb binding to nuclear material of polymorphs and marked the beginning of a rapidly evolving autoAb era in clinical diagnostics [6]. Indirect immunofluorescence (IIF) was the first assay technique employed to reveal autoAbs in patients with CTD [7]. The groundbreaking works of Holborow and Friou et al. led to

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the discovery of so-called antinuclear antibodies (ANAs) as marker autoAbs of CTD like SLE [8, 9]. In the following years, clinicians made tremendous efforts to understand the clinical significance of autoAbs and their potential use for the serological diagnosis of CTD and beyond [10]. This process was greatly driven by novel emerging assay techniques used for autoAb testing and their respective assay performance characteristics (Fig. 1; Table 1). The ensuing discourse has led to the definition of various diagnostic strategies for the serological diagnosis of autoimmune disorders and continues to date. Of note, ANA detected by IIF was included into the diagnostic criteria of SLE and autoimmune hepatitis (AIH) later [11–13]. In this context, the discovery of autoAbs to extractable nuclear antigens (ENAs) apart from autoAbs to dsDNA or histones in the search for disease-specific autoAbs provides an intriguing example for the change in the understanding of the clinical meaning of autoAbs as diagnostic markers [14–16]. Thus, the seminal paper of E.M. Tan and H.G. Kunkel on the identification of Sm as an autoantigenic target of SLE and the use of double radial immunodiffusion (DRID; Ouchterlony technique) for its detection ushered in a new era in autoAb diagnostics and its clinical application [17]. Although ANA turned out to be a sensitive marker for SARD as a whole disease group, its specificity for distinct SARD entities was not satisfactory despite being defined as a diagnostic marker for SLE [11]. Thus, the clinical need for more specific "ANA" was met by the pioneering work of H.G. Kunkel, E.M. Tan, and others discovering more and more novel autoAbs to ENA with clinical significance [14, 18]. However, not all ENAs identified as targets for CTD-specific autoAbs could be isolated by the saline extraction technique reported previously and should not be termed ENA [19]. Furthermore, apart from autoAbs recognizing nuclear autoantigens, anticytoplasmic autoAbs (ACyA) have been introduced into the autoAb panel for SARD serology [20]. Thus, the anti-SjS antigen A (SS-A) autoAbs also termed Ro have been shown to interact with its respective target in the cytoplasm [21]. As a fact, the progress in proteomics enabled the identification of cytoplasmic autoantigenic targets interacting with for instance myositis-specific autoAbs like anti-histidyl tRNAse autoAbs (Jo-1) or SLE specific autoAb against ribosomal proteins [22–24]. Obviously, this created confusion among clinical and laboratory experts and called for clarification. In terms of ANA testing, the introduction of human epidermoid laryngeal carcinoma (HEp-2) cells as improved autoantigenic substrate in IIF has encouraged the reporting of CTD-specific cytoplasmic patterns over the years [2]. This contradiction in terminology was addressed by a recent consensus recommending the use of anticellular antibodies instead of ANA [4]. Notwithstanding, the use of ANA and ENA is well established particularly among clinicians and it remains to be seen how this issue will be solved adequately in the years to come [25]. In summary, autoAb testing is an integral part in the serological diagnosis of CTD and may also assist in the prognosis, subclassification, as well as monitoring of disease activity [4, 10, 26–29].

As mentioned earlier, not only the discovery of novel SARD-specific autoAbs has challenged the diagnostic skills of clinicians but the introduction of novel assay techniques with differing assay performance, too [30]. Thus, the change from immunodiffusion-based detection techniques like DRID or counterimmunoelectrophoresis (CIE) detecting precipitating autoAbs to enzyme-linked immunosorbent assay (ELISA) regarding the analysis of autoAbs to Sm or SS-A called the specificity of these distinct markers suddenly into question [31–33]. The solid-phase ELISA brought about a significantly elevated sensitivity which in turn is related to a diminished diagnostic specificity [34]. Furthermore, with the better understanding of the chemical structure of for instance the small nuclear ribonucleoprotein (snRNP) complex representing the Sm autoantigen, six different protein structures (B, B', D, E, F, G) were identified as autoantigenic targets with SmD being apparently the most specific one for SLE [35–37]. Alone, these critical aspects require a comprehensive knowledge on

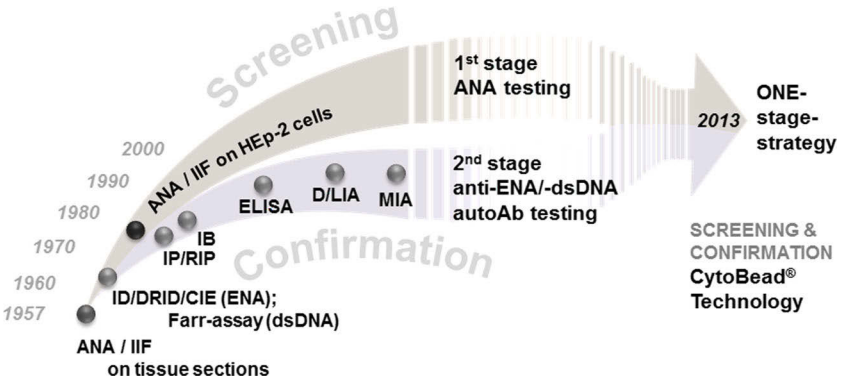

Fig. 1 Evolving autoantibody (autoAb) testing and strategies for the serological diagnosis of systemic autoimmune rheumatic diseases. *ANA* antinuclear antibody, *autoAb* autoantibody, *CIE* counterimmunoelectrophoresis, *D/LIA* dot/line immunoassay, *ELISA* enzyme-linked immunosorbent assay, *ENA* extractable nuclear antigen, *IB* immunoblot/westernblot, *ID/DRID* immunodiffusion/double radial immunodiffusion, *IIF* indirect immunofluorescence, *IP* immunoprecipitation, *MIA* microbead immunoassay, *RIP* radioimmunoprecipitation

Table 1 Autoantibody (AAB) detection methods in routine diagnostics of systemic rheumatic diseases

Method	Principle	Advantages	Disadvantages	Application
Chip technique (Spot immunoassay) [142, 168, 172–175]	autoAb binding to purified native or recombinant proteins immobilized as a spot on an adsorbent membrane, measurement: see ELISA	•More autoAb per test detectable compared to DIA/LIA •Very low amount of autoantigens needed	•Optimal epitope presentation for each autoantigen difficult to achieve •Possible interferences (see ELISA) may lead to false positive reactions	Multiparametric determination of autoAb
Crithidia luciliae Immunofluorescence Test [108, 110–113, 191]	In situ autoAb binding to kinetoplast DNA of *Crithidia luciliae*, visualization of autoAb binding by fluorescence-labeled anti-human IgG	High diagnostic specificity for SLE	•Low diagnostic sensitivity for SLE •Semiquantitative analyses only	Determination of dsDNA autoAb in suspicion of SLE or in sera with homogeneous ANA pattern
DIA/LIA [90, 116, 140, 151–159]	autoAb binding to purified native or recombinant proteins immobilized as dot or line on an adsorbent membrane, measurement: see ELISA	•Allows the specific detection of numerous autoAb per test including vary rare autoAb •Low amounts of autoantigens needed	•Qualitative or semi-quantitative analyses only •Possible interferences (see ELISA) may lead to false positive reactions	Multiparametric determination of autoAb (e.g., myositis or SSc specific autoAb)
Double radial immunodiffusion (Ouchterlony technique) [14, 16, 17, 21, 31, 90, 113]	Precipitation of the autoAb with the corresponding soluble autoantigen in gel after radial immunodiffusion; determination of autoAb specificity by reference antibodies	High diagnostic specificity for CTD	•Low diagnostic sensitivity for CTD •Time-consuming (24–48 h)	Screening for autoAb against ENA in serum of patients with suspected CTD
ELISA [3, 22, 32, 37, 53, 62, 71, 80, 95, 101, 120–122, 184]	autoAb binding to solid-phase (multiwell plate) immobilized autoantigen, measurement of autoAb interaction by enzyme-labeled anti-human IgG (or IgA, or IgM): colorimetry by substrate conversion with proportional behaviour to the strength of immune reaction	•Versatile and sensitive analytical technique •Good quantification •Good automation •Quick and cost-effective •Differentiation of immunoglobulin classes possible	Interferences may lead to false positive reactions (cross-reactive autoAb, matrix effects, endogenic proteins, nonspecific binding, autoAb against blocking proteins)	Specific determination of autoAb (highly purified native or recombinant autoantigens are required)
Farr radioimmunoassay [7–9, 55, 57, 67, 96, 106, 205]	Precipitation of anti-dsDNA/DNA complexes; Measurement of the quantity of dsDNA autoAb by using radioactively labeled dsDNA	•High diagnostic specificity for SLE •Superior for monitoring lupus disease activity compared to ELISA	•Requires radioactive material •Higher effort compared to ELISA	Specific detection and quantification of dsDNA autoAb
IIF on HEp-2 cells [7–9, 55, 57, 67, 96, 106, 205]	In situ autoAb binding to antigens of HEp-2 cells, visualization of autoAb binding by fluorescence molecule labeled anti-human IgG	•High sensitive detection of most clinically relevant nonorgan-specific autoAb •Optimal combination of immunoassays for further evaluation of specific autoAb taking into account IIF pattern and suspected diagnosis •Detection of diagnostically relevant autoAb without further need of specific immunoassays (e.g., centromere autoAb) •Assessment of autoAb only detectable by this method since the autoantigenic targets have not been identified or commercial assays are not available yet	•Subjective assessment •Reliable results require qualified and experienced lab personnel •High intralaboratory and interlaboratory variance → Automatic image recognition and interpretation improves and standardizes results	autoAb screening in sera of patients suspected of having SARD or autoimmune liver disease

Table 1 (continued)

Method	Principle	Advantages	Disadvantages	Application
Microparticle based immunoassays [102, 138, 139, 142, 164, 170–172, 176–179, 206–208]	autoAb bind to antigens immobilized on beads; measurement by flow cytometry (suspension bead assay) or optical microscope (planar bead assay)	•More autoAb per test detectable compared to DIA/LIA •Very low amount of autoantigens needed •Better epitope presentation for each autoantigen compared to spot assay •Combination with IIF possible (CytoBead® assay)	•Possible interferences (see ELISA) may lead to false-positive reactions	Multiparametric determination of autoAb
Passive agglutination (Latex test: RF) [216]	Binding of RF to human IgG bound on the surface of biologically inactive latex particles leads to visible agglutination of the particles	•Easy to perform •No need of instruments •High sensitivity	•Qualitative or semi-quantitative analyses only •False-positive reaction if reaction time is surpassed •Intensity of agglutination does not correlate with RF titer •Low specificity	Screening for RF (only rarely used in routine diagnostic since introduction of CCP autoAb)
Passive hemagglutination (Waaler-Rose test: RF) [217]	Binding of soluble autoantigens coated on red blood cells leads to visible erythrocyte agglutination	•Easy to perform •No need of instruments	•Qualitative or semi-quantitative analyses only •Subjective assessment	Not used anymore in routine diagnostics (in the past used for detection of RF, dsDNA, and Sm/RNP autoAbs)
Radioimmuno-precipitation assay [124, 125, 129]	autoAb binding to autoantigens of radiolabelled cell extracts; analyses of bound antigens by autoradiography after gel electrophoresis of the immunoprecipitates	Allows the detection of numerous autoAb without purification of autoantigens	•Requires radioactive material •Higher effort	Not used in routine practice; may be used for assay comparison and to search for novel autoAb (specialized labs only)
Westernblot (Immunoblot) [81, 89, 113]	autoAb binding to electrophoretically separated proteins transferred to adsorbent membrane, measurement: see ELISA	Allows the detection of numerous autoAb without purification of autoantigens	•False-negative results due to destroyed (denaturation of proteins during electrophoresis) or masked epitopes •False-positive results due to comigrated proteins	Not used anymore in routine diagnostics; may be used to search for novel autoAb
Nephelometry [218]	The amount of antigen/antibody complexes were measured by light scatter	•Easy to perform •Less-time consuming •Greater precision compared to latex test (see passive agglutination)	•No discrimination between isotypes •Lower diagnostic sensitivity compared to ELISA	Quantification of RF

ANA antinuclear antibody, *autoAb* autoantibody, *CCP* cyclic citrulinated peptide, *CTD* connected tissue disease, *DIA/LIA* dot/line immunoassay, *ELISA* enzyme-linked immunosorbent assay, *ENA* extractable nuclear antigen, *IIF* indirect immunofluorescence, *RF* rheumatoid factor, *SARD* systemic autoimmune disease, *SLE* systemic lupus erythematosus, *SSc* systemic sclerosis

the interpretation of assay characteristics by clinicians which were not always conveyed by laboratorians adequately [1, 3]. The badly needed comprehension of pretest and posttest probabilities of presence of disease and its relation to the diagnostic performance of autoAb analysis such as ANA testing appears not satisfactorily developed in clinicians [19, 38, 39]. Thus, novel diagnostic strategies translating the progress in autoAb testing proved difficult to get in line with established diagnostic pathways [27, 40, 41]. The recent attempt to substitute ANA IIF testing as screening assay within the two-tier strategy by novel multiplex techniques failed or met with great resistance among rheumatologists [4, 42, 43]. Consequently, the two-stage strategy recommending ANA testing by IIF as screening and appropriate confirmation of ANA positives by a different analysis was confirmed by expert consensus for CTD serology recently [4].

Autoimmune Vasculitis-Specific Autoantibodies

Of note, like revealed for the L.E. phenomenon in patients with SLE, patients suffering from autoimmune vasculitides demonstrate loss of tolerance to polymorphs, too [44]. In contrast, the occurring autoAbs recognize specific neutrophil cytoplasmic and not nonspecific nuclear components and were

described first in association with glomerulonephritis in 1982 by Davies et al. [45]. Van de Woude's group reported so-called antineutrophil cytoplasmic antibodies (ANCAs) to be associated with granulomatosis with polyangiitis (GPA, formerly Wegener's granulomatosis) shortly later and, consequently, the term ANCA-associated vasculitides (AAV) was coined [44, 46, 47]. Thus, this group of autoimmune vascular disorders comprises GPA, microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA, formerly Churg-Strauss syndrome) [48, 49]. Their leading clinical characteristics are microvascular inflammation, tissue necrosis, and the appearance of ANCAs [50].

Interestingly, similar to ANA testing, IIF was the first method to be used for the detection of ANCA revealing two major patterns, cytoplasmic (cANCA) and perinuclear ANCA (pANCA) [45, 51]. Not surprisingly, the respective main autoantigenic neutrophilic targets, proteinase 3 (PR3), and myeloperoxidase (MPO) were discovered shortly afterward [52, 53]. Consequently, a two-stage strategy for ANCA testing highlighting IIF as a standard method is recommended by international consensus for the serology of AAV, too [54]. Indeed, the unsurpassed high sensitivity of autoAb analysis employing cellular substrates by IIF renders this method an ideal tool for the screening stage followed by confirmatory testing with different immunological assay technologies [47]. However, similar to ANA IIF reading, interpretation of ANCA patterns is rather time consuming due to lack of automation and skilled laboratory experts [55]. Thus, IIF is in general highly subjective what renders appropriate standardization difficult [56, 57]. Therefore, attempts to replace IIF by novel techniques based on solid-phase immunoassays (e.g., ELISA, dot/line immunoassay, addressable bead/microarray assays) for ANCA as well as ANA analyses are increasing currently [58–62]. Indeed, in contrast to IIF, these assay techniques can be automated and proved to be more cost-efficient in the modern laboratory environment characterized by a rising diagnostic demand due to the growing clinical impact of autoimmune diseases. However, worrying rates of false-negative findings have been reported for these techniques in terms of ANA as well as ANCA testing [42]. Of note, this fact also appears to be relevant for organ-specific autoimmune disorders like celiac disease (CD).

Celiac Disease-Specific (Auto)Antibodies

Celiac disease, a gluten-related and immune-mediated small intestinal disease, is one of the few autoimmune disorders which the triggering factor was identified for [63]. Indeed, gliadin peptides deamidated by tissue transglutaminase type 2 (TG2) were shown as gluten-related T-cell epitopes triggering chronic inflammatory intestinal lesions and leading to villous atrophy and hyperplasia of the crypts [64].

Like for CTD and AAV, serology is paramount for the diagnosis of CD encompassing the detection of (auto)Abs to endomysium (EmA), deamidated gliadin peptides (DGP), and TG2 of the IgA isotype [65]. As a fact, due to the excellent assay performance of EmA testing by IIF, this particular autoAb is still considered the reference standard for CD-specific (auto)Abs [65–67]. However, similar to ANA and ANCA testing by IIF, EmA IIF analysis was questioned more and more because it may be subject to interobserver as well as substrate-related variability and is difficult to automate [68]. Obviously, testing of anti-TG2 autoAbs by immunometric solid-phase assays was favored instead [69–72].

In summary, IIF as one of the first techniques employed for autoAb testing in various autoimmune disease diagnostics appears to keep its appeal with laboratorians and clinicians despite several shortcomings [73, 74]. The integration of IIF as screening or standard method for autoAb analysis into two-stage or multiplex strategies was necessary as yet, but creates cost constraints for health care systems already burdened with spiraling costs. This calls for innovative solutions to meet the growing demand for autoAb testing in clinical routine.

Evolving Assay Techniques for Autoantibody Testing

Single Tests for autoAb Analysis

The introduction of fluorescent dyes and the development of immunochemical methods for the labeling of antibodies on the one hand and fluorescence microscopy on the other hand paved the way for IIF as powerful tool for autoAb analysis in the 1950s [75]. Thus, the detection of ANA by IIF employing first rodent liver tissue and later HEp-2 cells as autoantigenic substrate marks the beginning of autoAb detection in the serological diagnosis of CTD [7, 9, 76, 77]. However, it turned out soon that the clinical need for disease-specific autoAbs was not appropriately addressed by ANA testing alone. The search for more specific autoAbs led to the introduction of immunodiffusion techniques which enabled the discovery of disease-specific autoAbs like the Sm autoAb in patients suffering from SLE [16, 17, 21]. In particular, DID employing thymic extracts was used and clinicians learnt to appreciate the high specificity of this new parameter for CTD serology. Not surprisingly, autoAbs to Sm were included along with ANA in the diagnostic criteria for SLE later and are still considered as one of the most specific serological parameters for SLE [11, 14]. However, DRID is a time-consuming technique and, thus, was replaced by CIE enabling a faster and more sensitive detection of precipitating autoAbs later on [31]. Several other important autoAbs to the spliceosomal complex such as autoAbs to U1 ribonucleoprotein (U1-RNP) were identified in the quest for new CTD markers [21]. Anti-U1-RNP was established as a specific serological marker for

MCTD and found in patients with SLE as well [78, 79]. The introduction of new assay techniques like radio- (RIA) and enzyme immunoassays as well as radio/immunoprecipitation paved the way for the development of autoAb detection assays with better assay performance [32, 62, 80–86]. In particular, the progress in proteomics and the introduction of the immunoblot technique enabled the purification and identification of the distinct autoantigenic targets [33, 87–89]. It turned out that Sm and U1-RNP consist of several autoantigenic components including U1-RNA with different characteristics regarding their performance as split autoantigens especially in solid-phase ELISAs [79, 90, 91]. Furthermore, the SjS-specific autoantigens SS-A and SS-B form a complex interacting with yRNA [92]. Of note, this confers only to the SS-A 60 kDa unit whereas the 52 kDa SS-A (TRIM21) does not bind to yRNA and is not related to this snRNP complex [93, 94]. This raised the question of the best composition of these targets for the detection of the distinct autoAbs or the use of the target subcomponent with the best assay performance [90]. In terms of U1-RNP consisting of components A, C, and a 68 kDa polypeptide, it was found that at least two of these three should be used as solid-phase antigens to set up an appropriate ELISA for the detection of autoAbs to U1-RNP [19]. In contrast, SmD of the Sm complex with its six subcomponents mentioned earlier appeared to be the most specific and sensitive autoantigenic target in ELISA for the serology of SLE [14].

In general, the introduction of solid-phase assays like ELISA was accompanied by four major aspects changing the understanding of autoAb testing for CTD diagnostics: (i) a better usability as assay platform, (ii) an increasing sensitivity compared with immunodiffusion techniques, (iii) the different assay performance of autoAbs recognizing conformational or nonconformational, linear epitopes, and (iv) the introduction of reference sera for standardized diagnostics. This was an essential step toward standardization and automation of autoAb testing addressing the growing demand thereof due to the inclusion of autoAb testing into diagnostic or classification criteria of more and more autoimmune diseases and changed the autoimmune laboratory environment dramatically [80, 95]. Consequently, assay techniques like IIF, which have been prone to subjectivity and difficult to automate until recently, were subjected to a rising pressure to be substituted [73, 96, 97]. In this context, several researchers were tempted by the advantages of the ELISA technique and in particular its higher sensitivity to develop assays employing cellular extracts of MOLT4 or HEp-2 cells [98–101]. Furthermore, the elevated sensitivity of particularly anti-SS-A ELISAs revealed false-negative ANA sera of patients suffering from CTD [102–105]. Indeed, this seems to be the only autoantigenic target which is not adequately presented even by HEp-2 cells and can result in false-negative ANA findings by IIF. To overcome this shortcoming of the appreciated IIF technique, genetically modified HEp-2 cells with a higher expression of the SS-A 60 kDA polypeptide were introduced in ANA testing [103, 106].

Of note, the increased sensitivity of ELISA resulted in positive autoAb findings in nondiseased individuals, too, which started an intense discourse on the right method for cutoff determination [80]. Finally, receiver operating characteristics curve analysis was approved for quantitative methods like ELISA as the best approach to do so [39]. Part of the false-positive findings could be assigned to autoAbs occurring before the onset of disease as putative predictive markers thereof [29, 107]. Nonetheless, false-positive findings in ELISA could be a result of autoAbs to less disease-specific nonconformational epitopes [108]. These autoAbs often belong to the natural autoAb repertoire and display a low affinity to its corresponding targets [109]. A very convincing example is the anti-double-stranded DNA (dsDNA) autoAb which was established as diagnostic marker of SLE [110]. Of note, the SLE-specific dsDNA epitope is ill-defined and IIF assays employing kinetoplast dsDNA of *Crithidia luciliae* (CLIFT) with its characteristic epitope structure appear to provide the best specificity for this important disease activity-associated SLE marker [108, 111–113]. The replacement of CLIFT and the Farr RIA measuring mainly high-affinity anti-dsDNA autoAbs due to a high-salt reaction environment by ELISAs detecting autoAbs to both nonconformational and conformational dsDNA epitopes resulted in high numbers of false-positives particularly in patients with infectious diseases [114].

A similar phenomenon was observed when recombinant or synthetic autoantigens were introduced into autoAb testing to overcome the difficulties related to antigen purification and standardization [115, 116]. Not in each case, these nonnative polypeptides could replace the native autoantigenic targets for an appropriate autoAb analysis. Thus, the SmD polypeptide was dependent on the symmetric methylation of arginine to represent the SLE-specific epitope for the sensitive detection of anti-Sm autoAbs [35, 36, 117]. Furthermore, the presence of yRNA for the autoantigenicity of the SS-A/SS-B complex on the one hand and of U1-RNA for the Sm/RNP unit on the other hand was obviously required for the sensitive analysis of the respective autoAbs [118, 119].

Remarkably, specific ANCA testing demonstrated similar difficulties. Like for ANA testing, IIF was introduced as first assay technique on fixed neutrophils [45]. However, the following identification of PR3 and MPO as the main ANCA targets and the subsequent analysis of respective autoAbs by ELISA were hampered by the nonsatisfactory sensitivity of anti-PR3 autoAb tests [120–122]. Indeed, the conformational epitopes on PR3 were difficult to preserve on the solid phases of ELISAs. Recently, the third generation of PR3-ANCA ELISA has been introduced employing anchor molecules during adsorption of PR3 to the solid phase to preserve its confirmation and accessibility of vasculitis-specific epitopes [121, 122]. Other attempts to develop highly sensitive PR3-

ANCA ELISAs comprised the use of a mixture of native as well as recombinant PR3 [123].

The close relation between sensitivity and specificity is presumably the reason that direct-ligand RIAs with their excellent sensitivity have not been used widely for the analysis of CTD- or AAV-specific autoAbs. Interestingly, this is in contrast to organ-specific autoimmune entities such as type 1 diabetes (T1D) where RIAs are appreciated hitherto due to their high sensitivity [124, 125]. Of note, IIF on endocrine pancreas had also been the first technique used for autoAb analysis before the corresponding autoantigens were identified [126]. The detection of islet-cell autoAbs by IIF is still in use; however, the impact of conformational epitopes for T1D autoAbs testing in conjunction with the increased sensitivity of RIAs and recently emerging ELISAs with similar assay performances have almost replaced IIF [127].

After the discovery of TG2 as autoantigenic target of EmA for CD serology, a similar development was observed in the serological diagnosis of CD [128]. To obtain a sensitive anti-TG2 autoAb assay, conformational epitopes of TG2 seem to be essential, too [129]. In contrast to T1D autoAb testing, however, the detection of EmA by IIF is still the gold standard [65].

As a fact, the higher disease specificity of autoAbs to conformational epitopes is probably the reason for today's infrequent use of immunoblot assays for autoAb serology [130]. Obviously, due to the poor presentation of such epitopes on the blot membrane as a result of the denaturing effect of sodium dodecyl sulfate during electrophoresis and the poor standardization of the method due to technical peculiarities, the immunoblot technique has lost its initial appeal for multiplex autoAb testing [89, 131].

Notwithstanding, due to the progress in the identification of ever more autoAbs aiding in diagnosing, predicting and prognosing autoimmune diseases, the search for the most adequate strategy of autoAb testing fulfilling clinical needs and cost constraints has been in the focus of laboratory and clinical experts ever since [5, 59, 132–134]. For instance, more than 100 autoAbs were found in SLE patients alone [135]. This led to the introduction of fully automated random-access instruments employing fluorescence or chemiluminescence as read out for autoAb testing as well as screening [136–139].

Remarkably, a two-stage strategy was recommended for both ANA and ANCA analyses by international consensus recommendations [4, 54]. Thus, IIF is still considered a reliable screening test characterized by a high negative predictive value. Positive IIF findings should be confirmed by specific autoAb testing employing assay techniques with high specificity. For several other autoimmune disorders like for instance CD, IIF is still considered a gold standard [65]. Thus, despite the introduction of assay techniques for the detection of specific autoAb reactivities, there is still a need for testing of autoAbs by various assay techniques.

Multiplex Assays for autoAb Testing

The rising number of autoAbs requested for the serology of one autoimmune entity as well as the growing demand for autoAb testing in general encouraged the development of multiplex testing [3, 140–142]. Despite the fact that ANA assessment by IIF using HEp-2 cells as autoantigenic substrate is already a multiplex test revealing different patterns according to the autoAbs present in the serum investigated, the analysis of specific autoAbs is hardly achievable [20, 96, 143, 144]. Even for such ready to detect ANA patterns like the centromere one with its more than 40 fluorescent dots spread in nuclei of interphase cells and densely aligned dots in the metaphase cells, several proteins could be recognized by autoAbs as autoantigenic targets (centromere-associated proteins A, B, and C) [57, 76, 145–147].

As mentioned earlier, immunoblot was one of the first attempts to establish an appropriate multiplex test for the confirmation of ANA by using whole cell extracts with a similar autoantigen composition of HEp-2 cells [98, 148]. However, due to technical challenges, poor reproducibility, and loss of the native conformational structure of the relevant autoantigenic epitopes, this method was not established as a standard for multiplex autoAb analysis [19, 25, 149, 150].

As a result of improved purification methods for native autoantigens and progress in the expression of recombinant autoantigenic targets, the use of both molecule sources did not only enable the development of singleplex autoAb ELISAs but of multiplex dot or line immunoassays (D/LIAs), too [116, 140, 151]. In daily laboratory routine, D/LIAs have been established as one of the standard tests for ANA and ANCA confirmation [140, 152–154]. Moreover, D/LIAs appear to be an ideal solution for other serological diagnoses, where multiple autoAbs are required [155–158]. This holds not only true for CD serology where even a simultaneous IgA deficiency can be conducted apart from the (auto)Ab testing but proved to be very effective for the serology of SSc, DM/PM, or autoimmune liver diseases [140, 154]. Thus, D/LIAs with more than 20 autoantigenic targets have been introduced for the confirmatory diagnostics of ANA successfully [159]. Of note, the miniaturization of the technique by deploying sophisticated nanoliter dispensing devices and pattern recognition software for optical density reading render this technique most potential for future multiplex autoAb testing [160].

It should be noted in this context, that the attempts to employ the 96-well ELISA platform for autoAb multiplexing by using single wells for the immobilization of distinct antigens appear to be just an intermediate stage which was called into question very soon due to obvious shortcomings of the approach.

The progress in fluorescence reading as well as flow cytometry and microscopy paved the way for a new era in multiplexing [161–169]. Thus, several multiplex assay

developments employing surface-activated microbeads coded by fluorescent dyes, size, or shape on the one hand and fluorescence microscopy or flow cytometry as read-out on the other hand were reported [170–172].

The intriguing biochip mosaic technology enabled multiplex autoAb IIF reading by using various cellular and tissue substrates on one solid phase [173–175]. Further, the luminex technology deploying fluorescence-coded microbeads and flow cytometry enabled the development of an intriguing and very successful multiplex autoAb detection technique [176, 177]. Very soon, this novel technology was commercialized by several companies. The possibility to detect several autoAbs and the high throughput led to the development of such multiplex autoAb systems like Athena and FIDIS or the fully automated BioPlex2000 system covering various serological autoimmune diagnoses [172, 177–179]. The growing success and the ready automation of the luminex technology were very appealing especially for larger laboratories with ever growing sample volumes [177]. Indeed, demand for autoAb testing started rising exponentially in the 1980s and this phenomenon called into question even the recommended two-tier strategy encompassing IIF as the ideal autoAb screening [180–182]. As a matter of fact, laboratories in particular in the USA have begun replacing IIF due to its major shortcomings, namely lack of automation, standardization, modern data processing, and experts in IIF reading [3, 43, 73, 170, 183, 184]. Although the newly developed luminex applications for autoAb testing helped to ease the pressure in terms of rising autoAb analyses, there was growing dissatisfaction among rheumatologists with the assay performance of the technology [42]. Indeed, false-negative ANA findings leading to ill-defined diagnoses raised the concern of clinicians [185, 186]. Consequently, the American College of Rheumatology (ACR) initiated a task force in 2009 investigating the issue [42]. In conclusion, IIF was confirmed as standard method for ANA reading and laboratories requested to return to the two-stage strategy or to make sure that clinicians requesting ANA testing are aware of the different assay performance by multiplexing [4].

Of note, despite the development of similar multiplex tests for ANCA testing, IIF was also not challenged as screening assay in the two-stage strategy yet.

Improvement of IIF by Digital Fluorescence

The decision of the ANA task force of the ACR to retain the status of IIF and, thus, to confirm the two-stage strategy for CTD serology required an overhaul of the IIF technique badly [180, 181].

To employ this technique in a modern laboratory environment for CTD-associated antibody testing, the earlier mentioned shortcomings of IIF are needed to be addressed. In this context, the tremendous progress in fluorescence microscopy, image taking, and software development helped to usher in a new era of digital fluorescence [56, 161, 187, 188]. To the best of our knowledge, our group was the first to overcome critical disadvantages of ANA reading with IIF by introducing a standardized and automated fluorescence interpretation system which is based on the Videoscan technology and commercialized under the AKLIDES® brand [162, 189, 190]. AKLIDES® enables automated IIF reading by a sequential, multistage process including image acquisition by a CCD camera and software-controlled quality control, object segmentation, object description, and object classification by the use of novel pattern recognition algorithms. Thus, the system representing a composition of different hardware modules including a motorized inverse fluorescence microscope enables dynamic autofocusing resulting in the acquisition of quantitative fluorescence signals. The ensuing increased standardization and automation diminished the high intralaboratory and interlaboratory variability of ANA IIF reading, allowed the differentiation of cytoplasmic from nuclear staining, and rendered this method more applicable to high throughput screening [191–193].

Other diagnostic companies started developing similar systems and introducing new technologies for automated IIF pattern interpretation. In general, these commercially available systems are based on digital acquisition of fluorescence signals and most of them enable automated analysis of IIF images by pattern recognition algorithms (AKLIDES®, Medipan, Dahlewitz/Berlin, Germany; Nova View®, Inova, San Diego, USA; Zenit G Sight, A. Menarini Diagnostics, Grassina-Firenze, Italy; Europattern®, Euroimmun, Lübeck, Germany) [20, 194–196]. However, few systems distinguish between positive and negative screening results only (Helios, Aesku.Diagnostics, Wendelsheim, Germany; Image Navigator, Immuno Concepts, Sacramento, USA; Cytospot, Autoimmun Diagnostika, Straßberg, Germany) [185, 197]. In summary, all systems were reported to meet the demand for automated interpretation and satisfactory system performances were obtained by comparative studies at least for qualitative ANA evaluation [197, 198].

The fully automated interpretation system AKLIDES® was the first platform which performance was evaluated in clinical studies successfully [199–201]. Egerer et al. published the first clinical evaluation in 2010 by comparing the use of the new technology for ANA assessment of 1222 sera in the routine laboratory environment of both a university and a private referral laboratory [199]. An agreement of 93.0 % (859/924) and of 90.6 % (270/298) between automated AKLIDES® interpretation and classical ANA reading in the university

and the private laboratory were reported, respectively. Remarkably, end-titer analysis based on quantitative fluorescence reading was shown for the first time, which overcomes a crucial shortcoming of IIF and levels it with other quantitative assay techniques established in routine clinical laboratories. Thus, the application range of the novel interpretation systems (AKLIDES®, Europattern®, NovaView®) was enlarged by adding ANCA and anti-dsDNA autoAb testing on human neutrophils and *Crithidia luciliae*, respectively [191, 202–204].

In summary, the intriguing development of these novel automated IIF interpretation systems strengthened the position of IIF as screening technique within the two-tier strategy for ANA and ANCA analyses. Thus, the demand of even large laboratories in terms of automated autoAb testing by IIF with modern data management could be addressed adequately. Tozzoli et al. concluded that a new technological era in the routine autoimmune laboratory was reached by the introduction of fully automated IIF in 2009 [180]. Furthermore, this technology may also stimulate clinical research regarding larger population studies, e.g., the prevalence of the dense-fine speckled (DFS) pattern, and hence, of the DFS70 autoAbs, in different apparently healthy and diseased populations [205].

Combination of Screening and Confirmatory Testing

Irrespectively of the tremendous progress in automated autoAb testing by IIF at the beginning of this millennium, the constraint to use two different assay techniques for the recommended two-stage strategy of ANA and ANCA analyses has not been abolished yet [4]. This strategy enables a plausibility control of the obtained results because specific autoAb assays may give false-positive findings. For instance, a positive anti-dsDNA finding in ELISA in combination with ANA negativity cannot be regarded as relevant regarding diagnosis of SLE. However, the possibility of false-negative findings using the two-tier strategy especially for ANA reading in terms of sera positive for autoAbs to SS antigen A (SS-A/Ro) is still eminent at hand and represents an essential drawback of such approach [206]. Only the combination of both stages in one multiplex test would overcome these shortcomings and provide an ideal solution for autoAb testing addressing key clinical and laboratory needs. As a fact, this intriguing idea is quite simple, and thus, it appears astonishing that no such attempt was undertaken earlier. Hence, combination of the advantages of cell-based assays and the potential for multiplexing by microbead immunoassay (MIA) employing IIF within one reaction environment could revolutionize autoimmune diagnostics (Fig. 2).

Second-Generation ANA Testing

To realize the idea of combining autoAb screening and confirmation, we started developing a unique IIF reaction environment encompassing classical ANA analysis on HEp-2 cells and simultaneous multiplex detection of autoAbs by MIA. Indeed, merging screening and confirmatory testing for disease-specific autoAbs could generate many benefits including shorter hands-on times, better reproducibility of autoAb findings, and higher cost-effectiveness especially for larger sample series.

First, a MIA which utilizes multiple carboxylated polymethylmethacrylate bead populations differing in size and/or concentrations of fluorescent dye for multiplexing was developed [207]. The classification of bead populations and measurement of corresponding ligand fluorescence intensity was readily performed by AKLIDES® enabling the detection of six different antinuclear autoAbs to Scl-70, Sm, SS-A (Ro60), SS-B (La), CENP-B, and, dsDNA. This assay development created the basis for the design of a unique IIF reaction environment which could integrate the classical ANA testing on HEp-2 cells in one test [102]. The new assay technique combining classical ANA testing with confirmatory analysis by MIA was termed CytoBead® technology (Fig. 3a). Intriguingly, the novel options of digital fluorescence enabling quantitative analysis not only of specific autoAb testing by MIA but also of classical ANA reading on HEp-2 cells can be readily employed by CytoBead® assays. Thus, they can be standardized by calibrated interpretation systems for automated autoAb testing. Consequently, this is a new age of standardization of ANA testing as a whole which was not feasible with classical ANA testing by IIF in the past.

Altogether, a new generation of autoAb testing could be established that can meet the demand of modern routine service laboratories for the serology of SARD/CTD by addressing the key disadvantages of the currently recommended two-stage autoAb testing.

Recently, this new assay referred to as second generation ANA testing was evaluated in a comprehensive serological study comprising inter alia 174 patients with SLE, 103 with SSc, 46 with SjS, 36 with RA, 13 with MCTD, 21 with DM/PM, 21 with infectious disease, 93 with autoimmune liver diseases, 78 with inflammatory bowel disease, and 101 blood donors [102]. The CytoBead® ANA simultaneously determines ANA on HEp-2 cells and autoAbs to dsDNA, CENP-B, SS-A/Ro52, SS-A/Ro60, SS-B/La, RNP-Sm, Sm, and Scl-70. The obtained good agreement of the CytoBead® ANA with classical ANA reading by IIF and ELISA supports the notion that the novel combined reaction IIF environment for one-step ANA analysis employing HEp-2 cells and autoantigen-coated fluorescent beads as respective targets can provide at least the same assay performance like classical two-tier ANA testing.

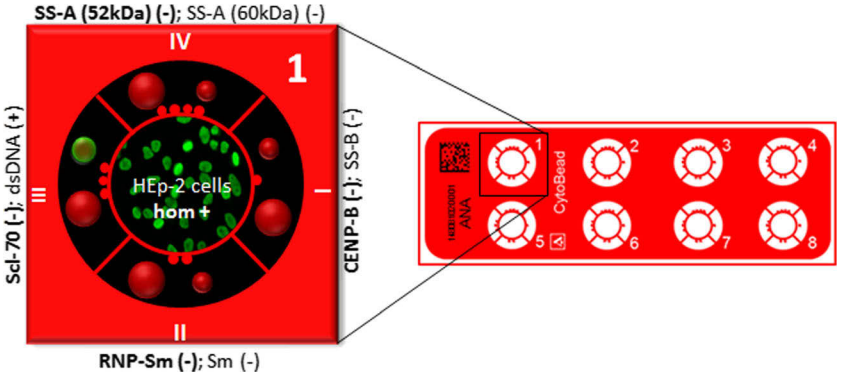

Fig. 2 Multiplexing strategy of CytoBead® technology exemplified for CytoBead® ANA assay. Combination of ANA screening with HEp-2 cells (*middle part*) and anti-ENA testing with antigen-coated microbeads (*peripheral parts I–IV*) in one reaction environment. Example of an ANA positive serum with positive homogeneous fluorescence pattern on HEp-2 cells and positive signal on dsDNA-coated microbeads presented as green fluorescence halo (*small red microbeads in part III*). *ANA* antinuclear antibody, *CENP* centromere protein, *Da* Dalton, *dsDNA* double-stranded DNA, *ENA* extractable nuclear antigen, *hom* homogeneous, *RNP* ribonuclear protein, *Scl-70* DNA-Topoisomerase I, *Sm* Smith, *SS* Sjögren-Syndrome, (+) positive, (−) negative

Furthermore, simultaneous detection of ANA and specific autoAbs such as to SS-A/Ro by CytoBead® ANA can almost eliminate the risk of false-negative findings and increase the already high negative predictive value of ANA testing. Of note, this is especially in the interest of rheumatologists who would like to exclude the presence of autoimmunity in their differential diagnosis of SARD by ordering ANA testing. In this study, 4/267 (1.5 %) ANA-negative patients with positive anti-SS-A or anti-CENP-B autoAbs were determined by second-generation ANA analysis. As a fact, these distinct patients with RA and SjS would have been missed by the currently recommended two-tier strategy since ANA negativity and positivity for anti-SS-A and anti-CENP-B autoAbs were confirmed by classical testing.

New-Generation ANCA Testing

The CytoBead® technology was also applied for the comprehensive analysis of ANCA and the resulting CytoBead® ANCA was evaluated in terms of its assay performance [208]. Indeed, the combination of both IIF and antigen-specific assays was found in several studies to be the optimal strategy for ANCA detection and led to the recommendation of a two-stage ANCA testing.

Alike CytoBead® ANA development, after having designed a multiplex addressable MIA detecting MPO-ANCA, PR3-ANCA, and autoAbs against the noncollagen region of the alpha-3 subunit of collagen IV representing the glomerular basement membrane (GBM) antigen, a unique reaction environment for the additional detection of ANCA on fixed neutrophils was generated (Fig. 3b). The novel CytoBead® ANCA is a unique combination of a classical cell-based assay with multiplexing microbead technology [204, 208].

Sowa et al. recruited 592 patients including 118 patients with AAV, 133 with RA, 49 with infectious diseases, 77 with inflammatory bowel disease, 20 with autoimmune liver diseases, 70 with primary sclerosing cholangitis (PSC), and 125 blood donors and compared multiplex CytoBead® ANCA testing with classical methods such as IIF and ELISA [208]. Quantitative PR3- and MPO-ANCA analysis by multiplex CytoBead® technology turned out to be at least equal or better compared to classical ELISA testing for specific ANCA. Remarkably, automated endpoint ANCA titer analysis by only one serum dilution employing the automated interpretation system AKLIDES® revealed a very good agreement with the classical ANCA IIF on neutrophils. Another intriguing finding was the detection of PR3-ANCA in patients suffering from ulcerative colitis (UC) and PSC apart from those with GPA. These data appear to confirm a recent report of PR3-ANCA positive patients suffering from UC and PSC detected by another sensitive MIA technique [138]. Thus, the new reaction environment of the CytoBead® ANCA enables highly sensitive PR3-ANCA testing and might compete with third-generation ELISA in terms of assay performance.

Consequently, automated multiplex IIF combining screening and confirmatory ANCA testing in one test may replace the time-consuming current two-stage ANCA testing strategy by a one-step multiplexing CytoBead® analysis [206]. In context of the emergency diagnostics required for rapidly progressive glomerulonephritis, the novel multiplex ANCA analysis by CytoBead® appears to be an attractive approach to meet the clinical need for comprehensive ANCA testing in the fastest way possible.

Comprehensive CD Serology

The serological diagnosis of CD comprises the detection of EMA and auto/Abs against deamidated gliadin and TG2 of the IgA isotype. As a fact, EmA detected by IIF is still

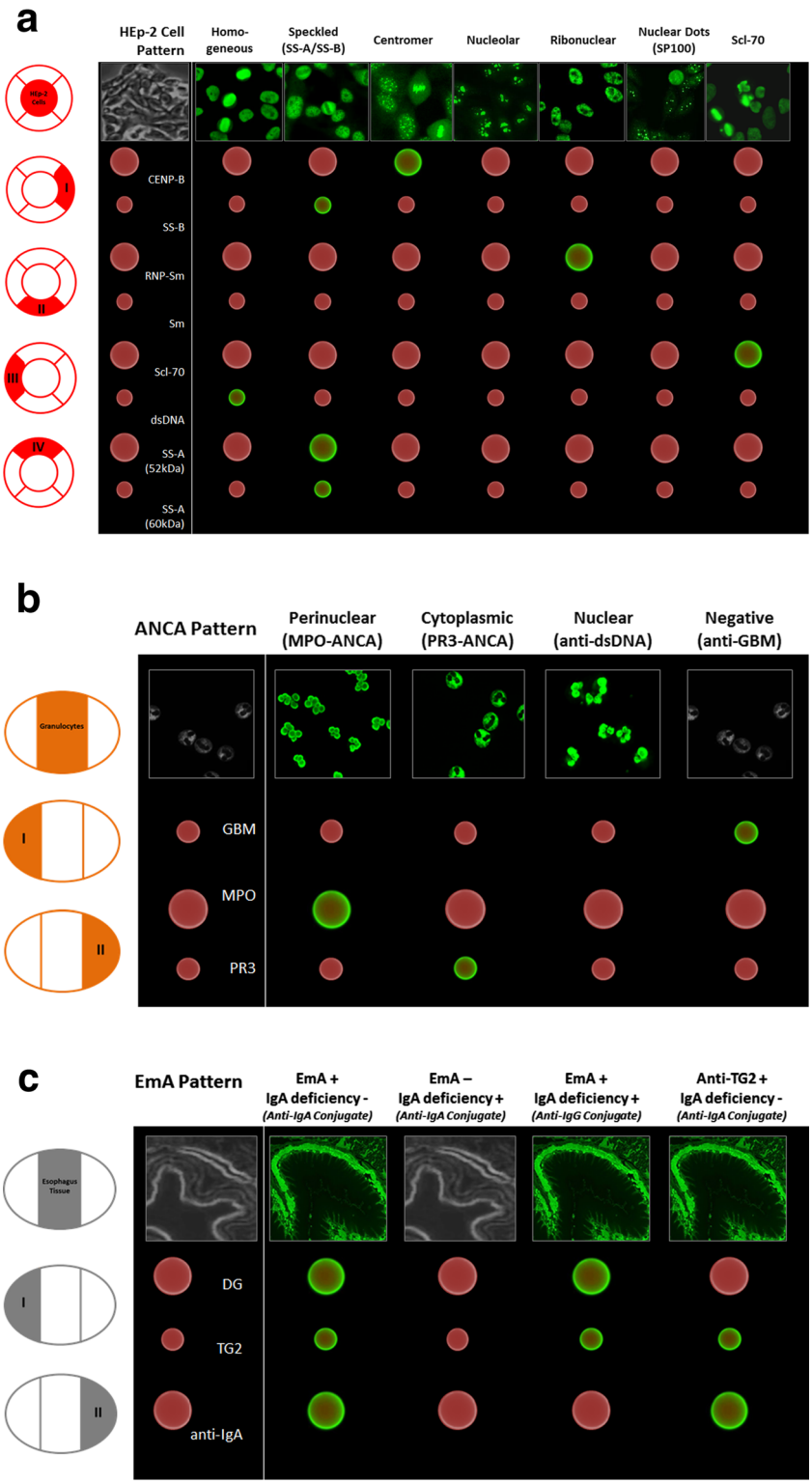

Fig. 3 CytoBead® assays for the detection of **a** antinuclear antibodies (ANA) with CytoBead® ANA assay, **b** antineutrophil cytoplasmic autoantibodies (ANCA) with CytoBead® ANCA assay, and **c** celiac disease (CD)-specific (auto)antibodies (auto/Abs) with CytoBead® CeliAK assay. Matching principle of specific fluorescence patterns on HEp-2 cells (**a**), neutrophil granulocytes (**b**), and esophagus tissue (**c**) with positive reactions of antigen-coated microbeads immobilized in peripheral compartments. *CENP* centromere protein, *Da* Dalton, *dsDNA* double-stranded DNA, *EmA* endomysial antibody, *GBM* glomerular basement membrane, *MPO* myeloperoxidase, *PR3* proteinase 3, *RNP* ribonuclear protein, *Scl-70* DNA-Topoisomerase I, *Sm* Smith, *SS* Sjögren-Syndrome, (+) positive, (−) negative

considered the gold standard for (auto)Ab testing in CD [65]. To address the need for comprehensive CD-specific (auto)Ab testing in terms of workload and cost reduction in routine autoimmune laboratories, we developed a multiplex CytoBead® CeliAK assay (Fig. 3c) [209]. Multiplex CD-specific (auto)Ab testing might even be an attractive diagnostic tool in the context of the novel diagnostic criteria published by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) recently [65]. These criteria obviously strengthen the role

of CD serology within the workup of patients with the suspicion of CD. Thus, CD can be diagnosed without histology by waiving duodenal biopsy in case of anti-TG2 autoAb IgA levels 10 times higher than the upper limit of normal (ULN) in patients positive for HLA-DQ2 or HLA-DQ8 and a positive response to gluten-free diet or confirmation by EmA testing.

Hence, the novel CytoBead® CeliAK was evaluated by investigating in total 380 patients and controls comprising 155 CD patients, 5 with IgA-deficiency, 68 with cystic fibrosis, 59 with eye diseases, and 93 blood donors [209]. Findings were compared with classical IgA-(auto)Ab analyses by ELISA and IIF. As a fact, the difference between CytoBead® and classical testing was only significant for anti-TG2 autoAb testing whereas the eight discrepant sera with anti-TG2 autoAb positivity by ELISA and negative levels by CytoBead® CeliAK belonged to four CD patients and four controls. Altogether, the CytoBead® CeliAK represents the first multiplex quantitative IgA anti-TG2 autoAb and anti-DG Ab multiplex assay which provides simultaneous EmA analysis as reference method and IgA deficiency testing. This comprehensive approach has the potential to improve CD serology and demonstrated excellent results regarding the great number of CD patients with anti-TG2 autoAb levels >10× ULN due to its high sensitivity. Additionally, due to the flexibility of the technique, further autoAbs such as those to GP2 stratifying CD patients further might be included [210, 211].

Conclusion

Hitherto, the history of autoAb testing has been characterized by an intriguing development of several assay techniques to keep up with the tremendous progress in the understanding of autoimmune diseases and their appropriate diagnostics [180, 197]. Today, autoAb analysis is an integral part in the serological diagnosis of SARD like CTD and AAV and organ-specific autoimmune disorders [4, 26, 60]. Hence, there is no doubt that the introduction and further evolvement of IIF as one of the first autoAb-detecting assay techniques had and have an essential impact on this process [162, 197]. In the history of autoAb testing, various techniques emerged and were replaced by newer ones providing better assay performance and benefits regarding higher sample throughput and standardization [34, 80]. In this context, it is astonishing to note that IIF is still one of the key techniques to analyze autoAbs and even recommended as screening assay within the two-stage strategy for ANA and ANCA testing. In addition, IIF remains a reference method for the detection of distinct autoAbs like EmA in the serology of organ-specific autoimmune disorders indeed [65].

Despite the obvious benefits of IIF, this assay technique has been characterized by time consuming and subjective evaluation, insufficient automation, as well as poor standardization since its introduction [162]. In particular, pattern reading for ANA and ANCA testing was prone to inconsistencies in description and classification of respective staining patterns.

As a consequence, novel assay techniques based on solid-phase immunoassays like ELISA or multiplexing technologies creating the basis for different commercial platforms evolved and were introduced into routine autoimmune laboratories [149]. Nonetheless, IIF is still recommended to be used as the gold standard method for instance for ANA testing due to the unsatisfactory assay performance of even the latest multiplex technologies in this important area of autoAb analysis [4].

This situation changed dramatically by the development of digital fluorescence and its implementation in IIF testing. The breathtaking new options of pattern recognition combined with progress in automated fluorescence microscopy paved the way for the evolvement of an entirely new generation of automated interpretation systems [206]. Different commercially available IIF platforms for autoAb testing were designed and applied for ANA and ANCA reading in particular. First evaluation studies support the good performance of these systems and high agreement between visual and automated autoAb interpretation [212].

Of note, this enormous technology development comprising digital fluorescence image acquisition and automatic pattern recognition could be extended to other cell-based IIF assays in the search for new biomarkers. Thus, the quantification of γH2AX foci for DNA damage analysis, which used to be time consuming, subjective, and not suitable for high-throughput screening, could be standardized and automated [213, 214]. Successful evaluation studies support the introduction of this new DNA damage marker into clinical routine for cytostatic resistance development diagnostics [215].

Nevertheless, since the majority of clinical immunology laboratories follow the two-stage strategy for ANA and ANCA testing, substantial constraints regarding high-throughput and cost-effectiveness remain. The expansion of automated IIF interpretation systems like AKLIDES® to assess addressable MIAs created a unique novel assay platform allowing fully automated evaluation of cell-based screening tests and antigen-specific multiplex assays in one reaction environment for the first time. The evolvement of the CytoBead® technology combining quantitative autoAb screening and confirmatory testing in one IIF analysis enables second-generation autoAb detection in one test. This intriguing multiplex reaction environment addresses key needs for an effective standardized autoAb testing in laboratory routine. Major disadvantages of classical autoAb analysis by IIF were overcome by this new technique. First diagnostic applications for second-generation ANA and ANCA testing as well as

comprehensive serology of CD-specific (auto)Abs were developed and successfully evaluated.

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Simultaneous comprehensive multiplex autoantibody analysis for rapidly progressive glomerulonephritis

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Abstract

Rapidly progressive glomerulonephritis (RPGN) is mainly caused by anti-glomerular basement membrane (GBM) antibody-mediated glomerulonephritis, immune-complex or anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides and leads to rapid loss of renal function. Detection of ANCA and autoantibodies (autoAbs) to GBM and dsDNA enables early diagnosis and appropriate treatment of RPGN aiding in preventing end-stage renal disease.

Determination of ANCA on neutrophils (ANCA) as well as autoAbs to myeloperoxidase (MPO-ANCA), proteinase 3 (PR3-ANCA), GBM, and dsDNA was performed by the novel multiplex CytoBead technology combining cell- and microbead-based autoAb analyses by automated indirect immunofluorescence (IIF). Forty patients with granulomatosis with polyangiitis (GPA), 48 with microscopic polyangiitis (MPA), 2 with eosinophilic GPA, 42 with systemic lupus erythematosus (SLE), 43 with Goodpasture syndrome (GPS), 57 with infectious diseases (INF), and 55 healthy subjects (HS) were analyzed and findings compared with classical single testing.

The CytoBead assay revealed for GPA, MPA, GPS, and SLE the following diagnostic sensitivities and for HS and INF the corresponding specificities: PR3-ANCA, 85.0% and 100.0%; MPO-ANCA, 77.1% and 99.1%; anti-GBM autoAb, 88.4% and 96.4%; anti-dsDNA autoAb, 83.3% and 97.3%; ANCA, 91.1% and 99.1%, respectively. Agreement with classical enzyme-linked immunosorbent assay and IIF was very good for anti-GBM autoAb, MPO-ANCA, PR3-ANCA, and ANCA, respectively. Anti-dsDNA autoAb comparative analysis demonstrated fair agreement only and a significant difference ($P = 0.0001$).

The CytoBead technology provides a unique multiplex reaction environment for simultaneous RPGN-specific autoAb testing. CytoBead RPGN assay is a promising alternative to time-consuming single parameter analysis and, thus, is well suited for emergency situations.

Abbreviations: AAV = ANCA-associated vasculitis, ANCA = anti-neutrophil cytoplasmic antibody, autoAb = autoantibody, CV = coefficient of variation, EGPA = eosinophilic granulomatosis with polyangiitis, ELISA = enzyme-linked immunosorbent assay, ethN = ethanol-fixed neutrophils, GPA = granulomatosis with polyangiitis, GPS = Goodpasture syndrome, HS = healthy subjects, IIF = indirect immunofluorescence, INF = infectious diseases, MFI = median fluorescence intensity, MPA = microscopic polyangiitis, ROC = receiver operating characteristic, RPGN = rapidly progressive glomerulonephritis, RT = room temperature, SLE = systemic lupus erythematosus, TIF = tagged image file.

Keywords: anti-neutrophil cytoplasmic antibody, digital fluorescence, immunoassay, microbead, rapidly progressive glomerulonephritis

1. Introduction

Rapidly progressive glomerulonephritis (RPGN) is a kidney syndrome clinically characterized by rapid decline of renal function, microscopic hematuria, mild (or non-nephrotic) proteinuria, and active urinary sediment. In patients with RPGN, the glomerular filtration rate decreases over a short period of time ranging in general from a few days to 3 months.[1] Light and electron microscopy analysis reveals glomerular crescent formation as the main histopathological finding in RPGN.[2,3] Specific autoantibody (autoAb) testing is an integral part of the serological diagnosis of RPGN and enables appropriate treatment to avoid progression to end-stage renal disease.[4,5]

From a pathological point of view taking into account the presence of autoAbs, RPGN can be stratified into 3 major groups: anti-glomerular basement membrane (GBM) autoAb disease (type I), immune complex disease (type II), and pauci-immune disease (type III).[6,7] Of note, a proper classification is difficult and many RPGN cases remain idiopathic.

Type I is caused by the deposition of autoAbs interacting with the noncollagenous region of the type IV collagen α_3 chain of GBM. When additional lung involvement occurs, this anti-GBM

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autoAb RPGN with pulmonary hemorrhage is named Goodpasture syndrome (GPS). RPGN patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) are classified as type III or pauci-immune because immune deposits are absent or scanty. Type III RPGN accounts for more than 50% of all RPGNs, especially in older ages. Of note, roughly 10% to 30% of patients with anti-GBM autoAb positivity demonstrate ANCA additionally, indicating a more progressive disease.[4,8–11]

Around 30% to 40% of patients suffering from RPGN have immune-complex disease due to the presence of systemic autoimmune rheumatic disease in particular systemic lupus erythematosus (SLE). Thus, patients suffering from SLE should be checked for renal involvement, because early detection and following appropriate treatment improves the renal outcome. Furthermore, 5% to 25% of patients with immune complex glomerulonephritis show ANCA positivity.[12–15]

Approximately 50% of RPGN patients suffer from pauci-immune disease whereas 80% to 90% of them have elevated ANCA levels. Of note, patients suffering from AAV, particularly those with progressive granulomatosis with polyangiitis (GPA) show renal involvement in most cases (70–77%).[4,16–18] Moreover, almost all patients suffering from other AAV like microscopic polyangiitis (MPA) show renal involvement.[4,19–21]

Patients with RPGN alone or those with additional pulmonary hemorrhage require immediate diagnosis and treatment due to the life-threatening prognosis.[22] Since clinical symptoms do not allow an appropriate differential diagnosis, fast analysis of above-mentioned autoAbs plays a pivotal role. For adequate ANCA testing, as a fact, the international consensus statement requires indirect immunofluorescence (IIF) on ethanol-fixed human neutrophils (ethN) confirmed by specific immunoassays for autoAbs to proteinase 3 (PR3-ANCA) and myeloperoxidase (MPO-ANCA).[18,23–26] All in all, up to 5 different tests with varying assay techniques should be performed to achieve a complete serological workup of patients with RPGN. Thus, a multiplex autoAb analysis combining these different techniques should be the method of choice.[27] To date, only the CytoBead technology enables such multiplex quantitative autoAb testing by digital IIF and automated IIF pattern interpretation.[28–30]

Consequently, a multiplex CytoBead assay was developed to determine ANCA on neutrophils, MPO-ANCA, PR3-ANCA, and autoAbs to GBM (anti-GBM) and dsDNA (anti-dsDNA) simultaneously in patients and controls. Findings were compared with classical testing by single assays.

2. Methods

2.1. Patients and controls

In total, 287 patients and controls, including 40 patients suffering from GPA, 48 from MPA, 2 from eosinophilic GPA (EGPA), 42 from SLE, 43 from GPS, 57 from infectious diseases (INF), and 55 healthy subjects (HS), were included into the study (Table 1). Specific laboratory tests for PR3- and MPO-ANCA as well as anti-GBM autoAb determination were performed in the Center of San Carlo Borromeo Hospital (Milan/Italy), where the patients were diagnosed and followed-up. Further, renal biopsies were performed on all GPS patients. Anti-dsDNA analysis was performed in Germany, Brandenburg-Technical University Cottbus-Senftenberg.

The diagnosis of clinical entities has been performed according to specific classification criteria.[25,26,31] The study was approved by the local ethics committee of Milano (CE Milano-Area B 8/7/2014, CS-GA-115565) and complies with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects and/or animals. Aliquots were stored at −20°C until used to detect antibody reactivity.

Table 1

Characteristics of patients and controls.

Diagnosis	N (F/M)	Median age (IQR)
ANCA associated vasculitis		
Granulomatosis with polyangiitis	40 (12/28)	58 (26)
Microscopic polyangiits	48 (30/18)	56 (22)
Eosinophilic granulomatosis with polyangiitis	2 (0/2)	73 (3)
Systemic autoimmune rheumatic disease		
Systemic lupus erythematosus	42 (35/7)	43 (18)
Infectious diseases		
HCV infection	25 (12/13)	74 (26)
HBV infection	3 (1/2)	33 (21)
EBV infection	3 (0/3)	26 (9)
Anti-mycoplasma positive	1 (0/1)	5 (0)
Undefined infectious disease	25 (16/9)	74 (22)
Rapidly progressive glomerulonephritis		
Goodpasture syndrome/anti-GBM nephritis	43 (20/23)	68 (22)
Healthy subjects	55 (5/50)	46 (14)

ANCA = anti-neutrophil cytoplasmic antibody, EBV = Epstein-Barr virus, F = female, GBM = glomerular basement membrane, HBV = hepatitis B virus, HCV = hepatitis C virus, IQR = interquartile range, M = male.

2.2. Determination of autoAb with antigen-specific ELISA

Specific autoAb to GBM (for GPS), PR3-ANCA (for GPA), and MPO-ANCA (for MPA) as well as dsDNA (for SLE) were detected using commercially available antigen-specific enzyme-linked immunosorbent assay (ELISA) (Phadia [Uppsala/Sweden], EuroDiagnostica [Lundavägen/Sweden] and GA Generic Assays GmbH [Dahlewitz/Berlin/Germany]). Assay performance was done according to the instructions of the manufacturers.

2.3. Detection of ANCA by IIF

The detection of ANCA (ethanol and formalin fixed) was performed by using a commercially available assay according to the instructions of the manufacturer.

2.4. Multiparametric autoAb detection with CytoBead technology

ANCA on ethN, MPO-ANCA, PR3-ANCA, and autoAbs to GMB and dsDNA were determined simultaneously by the CytoBead RPGN assay employing ethN from freshly donated human blood along with PR3 (human native), GBM antigen (human recombinant; type IV collagen α_3 chain,[32–34] MPO (human native), and dsDNA (salmon native) covalently linked to fluorescent microbeads of 9 and 15 μm (PolyAn, Berlin, Germany; excitation 610 nm/emission 690 nm) as autoantigenic targets on glass slides with compartmented wells (Fig. 1).[35] Fixation of neutrophils and immobilization of autoantigen-coated fluorescent beads was performed as described elsewhere.[30]

A serum dilution of 1/20 was incubated 30 min at room temperature (RT). After washing, secondary antihuman IgG conjugated to AlexaFluor488 in combination with 4′,6-diami-

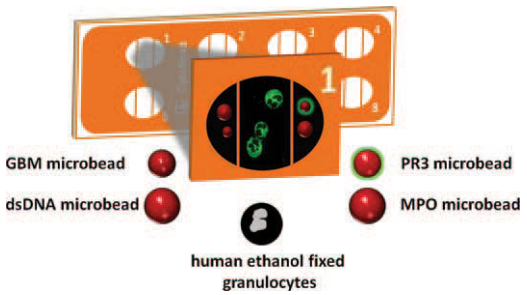

Figure 1. CytoBead RPGN glass slide for multiplex autoantibody (autoAb) analysis. Neutrophils isolated from donated human blood are fixed by ethanol in the middle compartment of each well for the detection of classical anti-neutrophil cytoplasmic antibodies (ANCA). Proteinase (PR3) and myeloperoxidase (MPO) are coated covalently on fluorescent microbeads of 9 and 15 μm, respectively, and immobilized on the right well compartment. Likewise, glomerular basement membrane (GBM) antigen and dsDNA are covalently linked to aforementioned microbead populations, respectively, and coated onto the left well compartment. The figure shows the reactivity pattern of a PR3-ANCA positive sample with a cytoplasmic fluorescence ANCA pattern on the neutrophils and a positive rim-like fluorescence signal on PR3-coated microbeads.

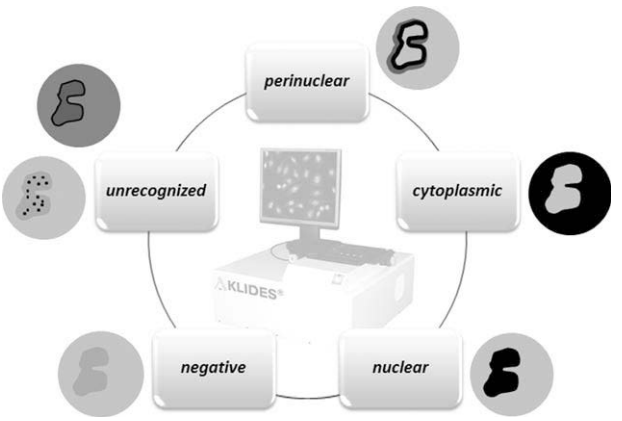

Figure 2. Anti-neutrophil cytoplasmic antibody (ANCA)-pattern recognition of indirect immunofluorescence (IIF) images on neutrophils by AKLIDES. The automated IIF interpretation system AKLIDES classifies cytoplasmic, perinuclear, nuclear, unrecognized, and negative fluorescence ANCA on ethanol-fixed neutrophils according to international guidelines[22,36,37,45].

dino-2-phenylindole was added and incubated for 30 min at RT, followed by a second washing step. Subsequently, slides were mounted either for automated evaluation with the IIF interpretation system AKLIDES (Medipan, Berlin, Germany) or manual analysis using a standard fluorescence microscope with green fluorescence channel (Carl Zeiss, Jena, Germany) as described elsewhere.[36–40] Fluorescence patterns of ethN were evaluated according to the international guidelines by AR.[25,26]

The final automated read-out was expressed in international units per mL (IU/mL) for PR3-ANCA, MPO-ANCA, and anti-dsDNA antibodies calibrated against the international reference sera of the Centers for Disease Control and Prevention (Serum 16 and 15, Atlanta, GA) and Wo/80, respectively. Furthermore, anti-GBM levels were determined in units per mL (U/mL) in accordance with internal standard material. All digital IIF images were captured and stored in lossless compressed tagged image file (TIF) format as reported earlier. Automated pattern recognition of ANCA IIF images was conducted as described elsewhere (Fig. 2).[30,37,38]

2.5. Analysis of coefficient of variation

Coefficient of variation (CV) was analyzed by using in-house reference sera. Each reference serum was diluted 3 times in order to get high, moderate, and low antibody concentrations.

Intra-assay CV was determined by 8 measurements for each serum while inter-assay CV was assessed by analyzing 8 determinations for each serum on 5 different days in accordance with the clinical and laboratory standards institute protocol EP15-A2. Microbead and ethN fluorescence analysis for the determination of median fluorescence intensity (MFI) was performed using AKLIDES.

2.6. Statistical analysis

The statistical analysis was performed by using MedCalc software (Version 12.4.0; MedCalc, Mariakerke, Belgium). Kruskal–Wallis test was used to compare unpaired cohorts. P values < 0.05 were considered statistically significant. Specific cut-off data were determined using receiver operating characteristic (ROC) curve analysis. Furthermore, inter-rater agreement (Cohen's kappa [κ]) and McNemar test were used for testing concordance values of CytoBead RPGN and routine test as well as clinical findings.

3. Results

3.1. Analysis of assay parameters

The cut-off determination of each parameter analyzed by the CytoBead RPGN was performed by ROC curve analysis employing patients with GPA, MPA, GPS, and SLE as disease groups for PR3-ANCA, MPO-ANCA, anti-GBM, and anti-dsDNA, respectively, and disease controls as well as HS as negative groups. Cut-offs were determined to match at least 95.0% specificity and revealed for PR3-ANCA, MPO-ANCA, anti-GBM, and anti-dsDNA values of 5 IU/mL, 5 IU/mL, 7 U/mL, and 10 IU/mL, respectively (Fig. 3). For ANCA testing by IIF pattern analysis on ethN, 70 MFI was used as cut-off as determined in an earlier study.[30]

Coefficients of variation (CVs) were determined using intra- and inter-assay datasets as described in "Methods" section. Intra- and inter-assay CVs of specific autoAb testing to PR3-ANCA, MPO-ANCA, GBM, and dsDNA showed values <15.0% which is in line with food and drug administration criteria Q2B (Table 2). Furthermore, intra-assay CVs of neutrophil cytoplasmic, perinuclear, and nuclear fluorescence staining patterns were also below 15.0% whereas corresponding inter-assay CVs exceeded 20.0% for 2/9 serum samples but were <23.0% altogether (Table 2).

3.2. ANCA and specific autoAb analysis by CytoBead RPGN

In total, 287 serum samples (Table 1) were analyzed for the presence of ANCA on ethN, PR3-ANCA, MPO-ANCA, anti-GBM, and anti-dsDNA by CytoBead technology. All 5 parameter levels demonstrated significant differences in the patient and control cohorts tested (Kruskal–Wallis test, $P < 0.005$, respectively; Fig. 4).

IIF testing on ethN by CytoBead RPGN revealed prevalences between 77.1% and 100.0% in patients with SLE, GPA, MPA, and EGPA (Table 3). In contrast, HS and INF demonstrated prevalences of 0.0% and 1.7% only, respectively. Interestingly,

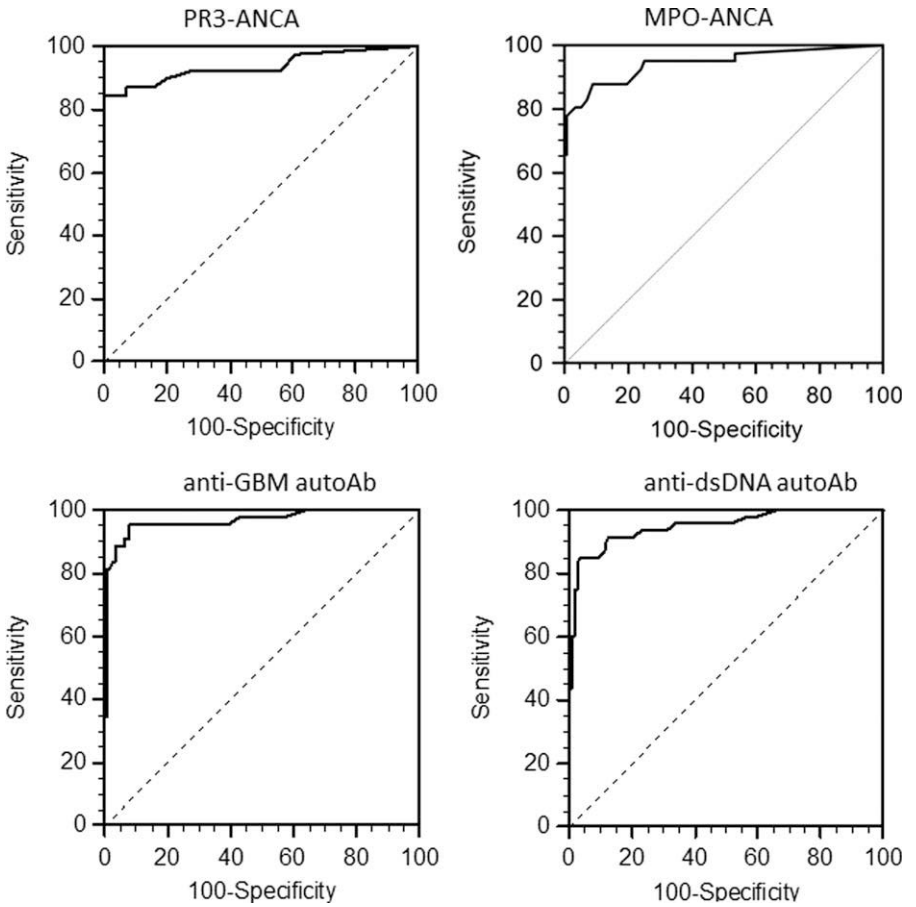

Figure 3. Receiver operating characteristic curve analysis for the determination of cut-off values of proteinase 3 (PR3)-anti-neutrophil cytoplasmic antibody (ANCA), myeloperoxidase (MPO)-ANCA, anti-glomerular basement membrane (GBM), and anti-dsDNA autoantibodies (autoAb).

Table 2

Intra- and inter-assay variation of (A) ANCA and (B) PR3-ANCA, MPO-ANCA, anti-GBM, and anti-dsDNA autoantibodies (autoAb) by CytoBead RPGN.

(A) ANCA	Cytoplasmic ANCA			Perinuclear ANCA		
Serum titer	High	Moderate	Low	High	Moderate	Low
Intra-assay CV, %	10.8	4.0	12.1	3.3	1.8	3.1
Inter-assay CV, %	19.9	19.9	22.3	15.1	12.1	7.8

	Nuclear autoAb		
Serum titer	High	Moderate	Low
Intra-assay CV, %	6.8	10.9	13.2
Inter-assay CV, %	12.6	19.2	22.7

(B) Specific autoAb	PR3-ANCA			MPO-ANCA		
Serum titer	High	Moderate	Low	High	Moderate	Low
Intra-assay CV, %	0.8	6.6	13.4	0.4	14.9	14.4
Inter-assay CV, %	2.2	1.4	8.5	9.5	10.1	13.1

	Anti-dsDNA autoAb			Anti-GBM autoAb		
Serum titer	High	Moderate	Low	High	Moderate	Low
Intra-assay CV, %	6.1	14.5	9.8	4.1	2.7	14.2
Inter-assay CV, %	10.8	10.1	10.0	13.4	14.5	14.4

ANCA = anti-neutrophil cytoplasmic antibody, CV = coefficient of variation, GBM = glomerular basement membrane, MPO = myeloperoxidase, PR3 = proteinase 3, RPGN = rapidly progressive glomerulonephritis.

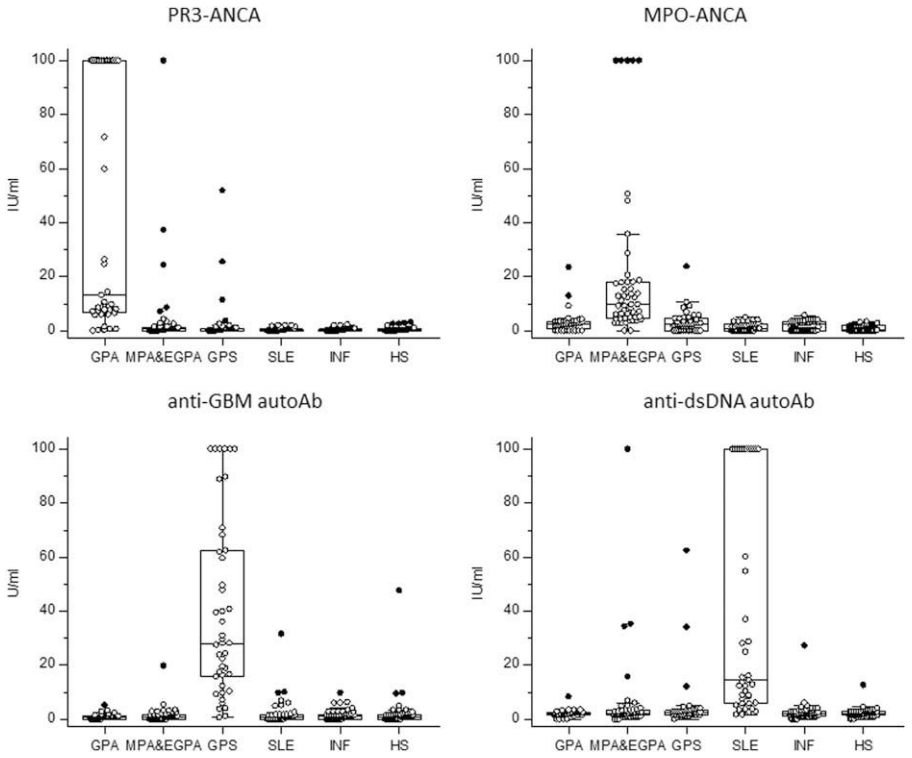

Figure 4. Detection of autoantibodies (autoAb) to dsDNA, glomerular basement membrane (GBM), myeloperoxidase (MPO), and proteinase 3 (PR3)-anti-neutrophil cytoplasmic antibody (ANCA) by CytoBead RPGN. Indirect immunofluorescence findings were interpreted on the automated interpretation system AKLIDES. EGPA = eosinophilic granulomatosis with polyangiitis, GPA = granulomatosis with polyangiitis, GPS = Goodpasture syndrome, HS = healthy subjects, INF = infectious diseases, MPA = microscopic polyangiitis, RPGN = rapidly progressive glomerulonephritis, SLE = systemic lupus erythematosus.

14/43 (32.6%) patients with GPS showed ANCA reactivity, of which 10 were confirmed by specific autoAb testing.

Specific autoAb testing by CytoBead RPGN demonstrated prevalences of 85.0%, 77.1%, 88.4%, and 83.3% for PR3-ANCA, MPO-ANCA, anti-GBM, and anti-dsDNA in patients with GPA, MPA, GPS, and SLE, respectively (Table 3). In contrast, HS showed prevalences between 0.0% and 5.4% and INF between 0.0% and 3.5% regarding these specific autoAbs.

3.3. Comparison of classical ANCA analysis with CytoBead RPGN testing

The performance of the multiplex CytoBead RPGN was further evaluated by comparison with classical ANCA testing by IIF and specific autoAb determination by solid-phase immunoassays. Consequently, 287 serum samples were analyzed with CytoBead RPGN and with classical tests employing IIF with ethanol and formalin-fixed neutrophils as well as specific ELISA (Table 4). Inter-rater agreement showed very good agreement for anti-GBM autoAb, PR3-ANCA, and MPO-ANCA and fair agreement for anti-dsDNA autoAbs (Table 4).

As a fact, comparison of anti-dsDNA autoAb testing revealed 52/287 (18.1%) discrepant results. Thus, whereas anti-GBM autoAb, PR3-ANCA, and MPO-ANCA comparative analysis of both methods did not reveal significant differences (McNemar test, $P > 0.05$, respectively), testing of anti-dsDNA autoAb did (difference 12.54%, 95% confidence interval: 7.94–15.62, $P <$

Table 3

Prevalence of ANCA by IIF, PR3-ANCA, MPO-ANCA, anti-GBM, and anti-dsDNA autoantibodies by CytoBead RPGN, classical testing employing ELISA and IIF on ethanol and formalin-fixed neutrophils, in 287 patients and controls.

	CytoBead RPGN, %						Classical tests, %					
Cohorts	**PR3-ANCA**	**MPO-ANCA**	**Anti-GBM**	**Anti-dsDNA**	**ANCA**	**Bead + IIF**	**PR3 ELISA**	**MPO ELISA**	**GBM ELISA**	**dsDNA ELISA**	**ANCA**	**ELISA + IIF**
GPA (n = 40)	**34 (85.0)**	3 (7.5)	0 (0.0)	1 (2.5)	35 (87.5)	33 (82.5)	**32 (80.0)**	3 (7.5)	0 (0.0)	7 (17.5)	39 (97.5)	35 (87.5)
MPA (n = 48)	4 (8.3)	**37 (77.1)**	0 (0.0)	6 (12.5)	45 (93.8)	38 (79.2)	4 (8.3)	**42 (87.5)**	0 (0.0)	17 (35.4)	47 (97.9)	44 (91.7)
EGPA (n = 2)	0 (0.0)	2 (100.0)	1 (50.0)	0 (0.0)	2 (100.0)	2 (100.0)	0 (0.0)	2 (100.0)	0 (0.0)	1 (50.0)	2 (100.0)	2 (100.0)
GPS (n = 43)	3 (6.9)	7 (16.3)	**38 (88.4)**	3 (6.9)	14 (32.6)	13 (30.2)	1 (2.3)	7 (16.3)	**41 (95.3)**	6 (14.0)	9 (56.25)	10 (23.3)
SLE (n = 42)	0 (0.0)	0 (0.0)	3 (6.25)	**35 (83.3)**	34 (81.0)	31 (73.8)	0 (0.0)	0 (0.0)	0 (0.0)	**36 (85.7)**	34 (81.0)	27 (64.3)
INF (n = 57)	0 (0.0)	1 (1.7)	1 (1.7)	2 (3.5)	1 (1.7)	1 (1.7)	0 (0.0)	0 (0.0)	0 (0.0)	14 (24.6)	1 (1.7)	2 (3.5)
HS (n = 55)	0 (0.0)	0 (0.0)	3 (5.4)	1 (1.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (5.4)	0 (0.0)	0 (0.0)

ANCA = anti-neutrophil cytoplasmic antibody, EGPA = eosinophilic granulomatosis with polyangiitis, ELISA = enzyme-linked immunosorbent assays, GBM = glomerular basement membrane, GPA = granulomatosis with polyangiitis, GPS = Goodpasture syndrome, HS = healthy subjects, IIF = indirect immunofluorescence, INF = infectious diseases, MPA = microscopic polyangiitis, MPO = myeloperoxidase, PR3 = proteinase 3, RPGN = rapidly progressive glomerulonephritis, SLE = systemic lupus erythematosus.

Table 4

Comparison of ANCA by IIF, PR3-ANCA, MPO-ANCA, anti-GBM, and anti-dsDNA autoantibody (autoAb) analysis by CytoBead RPGN and classical testing employing ELISA in 287 patients and controls.

		CytoBead RPGN						
		PR3-ANCA				MPO-ANCA		
		Negative	Positive			Negative	Positive	
PR3-ANCA ELISA	Negative	243	7		MPO-ANCA ELISA	230	9	
	Positive	3	34			7	41	
				∑ 287				∑ 287
Weighted kappa	0.852				Weighted kappa	0.803		
Standard error	0.046				Standard error	0.047		
95% CI	0.762–0.941				95% CI	0.710–0.896		

		CytoBead RPGN						
		Anti-GBM autoAb				Anti-dsDNA autoAb		
		Negative	Positive			Negative	Positive	
GBM ELISA	Negative	237	9		dsDNA ELISA	195	8	
	Positive	4	37			44	40	
				∑ 287				∑ 287
Weighted kappa	0.824				Weighted kappa	0.500		
Standard error	0.047				Standard error	0.057		
95% CI	0.731–0.917				95% CI	0.387–0.612		

ANCA = anti-neutrophil cytoplasmic antibody, CI = confidence interval, ELISA = enzyme-linked immunosorbent assays, GBM = glomerular basement membrane, IIF = indirect immunofluorescence, MPO = myeloperoxidase, PR3 = proteinase 3, RPGN = rapidly progressive glomerulonephritis.

0.0001). The 8 positive anti-dsDNA autoAb findings by CytoBead RPGN and negative with solid-phase immunoassays belong to patients with SLE (3, 37.5%), GPS (2, 25%), INF (1, 12.5%), and HS (1, 12.5%). Only 3/44 (6.8%) sera with negative anti-dsDNA autoAbs by CytoBead RPGN and positive test results by solid-phase assays are from patients with SLE. The further discrepant 41 disease and healthy controls of this particular group contain 13 patients with INF and 11 with MPA. Of note, CytoBead RPGN revealed only 1 false positive each regarding the respective discrepant control patient groups.

Findings of the AKLIDES software for automated pattern recognition showed very good agreement ($\kappa = 0.885$) with manual reading by an expert in ANCA diagnostics (Table 5).

4. Discussion

A patient with RPGN suffering from selective or combined kidney and lung disease is classified as clinical emergency case and has to be treated very fast to avoid fatal progression of disease. In particular, patients with GPS are identified to have the worst prognosis of all RPGN patients without the correct medical treatment.[2] As a matter of fact, in such critical settings, autoAb analysis is crucial for diagnosing patients adequately. Thus, determination of anti-GBM autoAbs for GPS, ANCA for ANCA-associated RPGN, and autoAb to dsDNA are recommended for an appropriate serological diagnosis of RPGN.[34,41–45] However, the analysis of all these parameters requires different techniques and is time consuming. Hence, there is a need for 1 step multiplex analysis addressing the urgent need for express RPGN serology.

In this context, the present study evaluated the multiparametric assay CytoBead RPGN for the simultaneous analysis of ANCA on ethN, MPO-ANCA, PR3-ANCA, and autoAbs to GBM and dsDNA.

With regard to ANCA pattern interpretation, the majority of patterns interpreted by AKLIDES were in line with the findings of a human expert. The AKLIDES system gives the result "unrecognized," when the pattern is not a classical cytoplasmic, perinuclear, or nuclear one, thus further interpretation by an expert is possible using the saved TIF images afterwards.[30,46] In that case, the "unrecognized" pattern could be declared as atypical or classified as perinuclear, cytoplasmic, or nuclear. The very good concordance of automated and manually obtained fluorescence patterns in this study might provide the basis for a

Table 5

Comparison of automated and manual ANCA pattern evaluation by the AKLIDES system and an expert in ANCA diagnostics.

	AKLIDES system				
Manual	**Perinuclear**	**Cytoplasmic**	**Nuclear**	**Unrecognized**	**Negative**
Perinuclear	**36**	1	3	5	0
Cytoplasmic	3	**47**	2	3	2
Nuclear	0	0	**29**	0	0
Unrecognized	5	1	1	**10**	0
Negative	0	0	0	0	**145**

Fluorescence patterns were categorized according to international guidelines (25, 26, 30, and 46). The inter-rater agreement (Cohen's kappa) of the different evaluation strategies was very good 0.885. Weighted kappa: 0.885; standard error: 0.023; 95% CI: 0.841–0.93.
CI = confidence interval.

Table 6

Comparison of diagnostic performance parameters of autoantibodies detected by CytoBead RPGN in patients with GPA, MPA, anti-GBM nephritis/GPS, and SLE.

	Disease	Prevalence, %	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	+LR	−LR
PR3-ANCA	GPA	13.9	85.0 (70.2–94.3)	97.2 (94.3–98.9)	82.9 (67.9–92.9)	97.6 (94.8–99.1)	30.0 (14.28–63.0)	0.2 (0.1–0.3)
MPO-ANCA	EGPA and MPA	17.4	78.0 (64.0–88.5)	95.4 (91.9–97.7)	78.0 (64.0–88.5)	95.4 (91.9–97.7)	16.8 (9.3–30.5)	0.2 (0.1–0.4)
Anti-GBM	GPS	15.0	88.4 (74.9–96.1)	96.7 (93.6–98.6)	82.6 (68.6–92.2)	97.9 (95.2–99.3)	26.95 (13.5–53.7)	0.1 (0.1–0.3)
Anti-dsDNA	SLE	15.2	83.3 (68.6–93.0)	94.5 (90.7–97.0)	72.9 (58.2–84.7)	96.9 (93.8–98.8)	15.06 (8.7–26.0)	0.2 (0.1–0.4)

PR3-ANCA, MPO-ANCA, autoAb to anti-GBM, and anti-dsDNA were determined by CytoBead RPGN. Disease prevalence reflects the prevalence of the given disease in the whole study cohort.
−LR = negative likelihood ratio, +LR = positive likelihood ratio, ANCA = anti-neutrophil cytoplasmic antibody, CI = confidence interval, GBM = glomerular basement membrane, GPA = granulomatosis with polyangiitis, GPS = Goodpasture syndrome, MPA = microscopic polyangiitis, MPO = myeloperoxidase, NPV = negative predictive value, PPV = positive predictive value, PR3 = proteinase 3, RPGN = rapidly progressive glomerulonephritis, SLE = systemic lupus erythematosus.

successful introduction of automated ANCA reading into routine diagnostics of RPGN and AAV. The present data are corroborated by recent reports demonstrating the usefulness of the novel pattern recognition algorithms used by the automated interpretation system AKLIDES for ANCA reading.[30,46]

Digital fluorescence enables standardization and quantitative end-point titer reading for autoAb testing for the first time in autoimmune diagnostics and, thus, offers new exciting perspectives with regard to automation and multiplexing.[36–39]

For rapid simultaneous multiparametric quantitative determination of several specific RPGN-specific autoAbs, antigen-coated fluorescent microbeads, and lot-specific calibration curves fitted by asymmetric 5-parameter equations were employed.[28–30] Of note, obtained diagnostic parameters for MPO-ANCA, PR3-ANCA, anti-GBM, and anti-dsDNA autoAbs in MPA, GPA, GPS, and SLE matched literature data adequately (Table 6).[41,42,45,47–50] In fact, anti-dsDNA antibody detection by CytoBead RPGN showed a diagnostic sensitivity of 83.3% with a diagnostic specificity of 97.3% in patients with SLE compared to a routine ELISA used by default in the nephrology department demonstrating 85.7% diagnostic sensitivity along with a poorer diagnostic specificity of only 84.8%.[47] Indeed, comparative anti-dsDNA autoAb analysis revealed a significant difference for both techniques and a fair agreement only. As a fact, CytoBead RPGN determined significantly less false-positive anti-dsDNA autoAb findings compared to ELISA.

The better specificity of the CytoBead RPGN anti-dsDNA autoAb detection might be a result of the specific covalent coupling strategy of the complete and nonfragmented dsDNA molecules to the activated microbead surface.[6] In addition, the agreement of anti-GBM autoAb, PR3-ANCA, and MPO-ANCA testing by CytoBead RPGN with classical corresponding ELISA was very good.

These findings support the assumption that the CytoBead RPGN is an attractive alternative to classical single testing regarding the analysis of all diagnostic relevant antibody specificities for the correct serological diagnosis of RPGN variants.

Furthermore, CytoBead RPGN is characterized by a very low incubation time of 1 h in contrast to current single routine tests. Hence, treatment of RPGN patients could start much earlier by addressing the most critical limiting factor for patients well-being or even survival.

Another characteristic of the CytoBead RPGN assay is its flexibility with regard to the autoimmune laboratory. Indeed, the assay can be run manually and interpreted by a conventional fluorescent microscope for qualitative autoAb assessment. Thus, emergency diagnostics for RPGN can be run without the need of expensive equipment by retaining all the benefits of multiplex autoAb analysis.

Our study has certain limitations. HS are not age and gender matched with the study cohorts. Further, the relevant prevalences of the disease cohorts do probably not reflect the actual prevalences in most nephrology departments. In order to obtain quantitative data for further evaluation, an automated interpretation system would have been necessary.

5. Conclusions

The multiparametric CytoBead technology is a unique combination of screening and confirmatory autoAb testing for RPGN serology and might be a very promising alternative to classical time-consuming single parameter testing. In the present study, CytoBead RPGN demonstrated satisfactory assay performance of the multiplex reaction environment for the detection of ANCA, PR3-ANCA, MPO-ANCA, autoAb to dsDNA and GBM addressing the need for emergency testing in routine autoimmune laboratories.

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Mucosal Autoimmunity to Cell-Bound GP2 Isoforms Is a Sensitive Marker in PSC and Associated With the Clinical Phenotype

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Introduction: Zymogen granule glycoprotein 2 (GP2) was demonstrated as first autoimmune mucosal target in primary sclerosing cholangitis (PSC) associated with disease severity. Autoantibodies to four GP2 isoforms ($aGP2_{1-4}$) were found in patients with inflammatory bowel diseases but reactivity against specific GP2 epitopes has not been investigated in PSC yet. Hence, the prevalence of $aGP2_{1-4}$ and their association with the PSC phenotype for risk prediction were examined.

Methods: GP2 isoforms were stably expressed as glycosylphosphatidyl -inositol-anchored molecules in the membrane of HEp-2 cells and used as autoantigenic targets in indirect immunofluorescence assay (IFA). $aGP2_{1-4}$ IgA and IgG were detected by IFA in 212 PSC patients of four European university hospitals and 145 controls comprising 95 patients with cystic fibrosis and 50 healthy subjects.

Results: Combined $aGP2_1$ and $aGP2_4$ IgA testing with a sensitivity of 66.0% and a specificity of 97.9% resulted in the best diagnostic performance (Youden index: 0.64) regarding all aGP2 and combinations thereof. $aGP2_4$ IgA positivity is significantly associated with the presence of cirrhosis in PSC ($p = 0.0056$). Logistic regression revealed the occurrence of $aGP2_1$ IgA (odds ratio [OR] 1.38, 95% confidence interval [CI]: 1.03–1.86) and $aGP2_4$ IgA (OR 1.52, 95%CI: 1.07–2.15) along with male gender (OR 0.51, 95%CI: 0.27–0.97) and older age (OR 1.03 95%CI: 1.01–1.05) as significant risks for the concomitant presence of cirrhosis in PSC.

Conclusions: Combined aGP2$_1$ and aGP2$_4$ IgA analysis is preferred to single aGP2 isoform analysis for sensitive PSC autoantibody testing. Positivity for aGP2$_1$ and aGP2$_4$ IgA is associated with cirrhosis in PSC and could be used for risk stratification.

Keywords: zymogen granule glycoprotein 2, primary sclerosing cholangitis, cirrhosis, cholangiocarcinoma, immunoglobulin A

INTRODUCTION

Primary sclerosing cholangitis (PSC), a chronic immune-mediated, life threatening, genetically predisposed, cholestatic liver illness, is associated with the co-occurrence of inflammatory bowel disease (IBD) and in particular with the phenotype thereof (1, 2). The prevalence of PSC is estimated at up to 16.2 per 100,000 individuals and still rising (3, 4). There is a clinical need for markers predicting PSC severity and prognosis despite recent progress regarding the use of certain serum and bile proteins (5, 6). Further, IgA to zymogen granule glycoprotein 2 (GP2) was identified as a novel marker candidate for disease severity and cholangiocarcinoma in PSC (7, 8). Glycoprotein 2 is a microbe-sensing (9, 10) and immunomodulating molecule (11) with two major sources (pancreas and intestine) (12). Of note, GP2 was originally identified as a target of Crohn's disease (CD)-specific pancreatic antibodies (13, 14). Upon specific binding to FimH, GP2 interacts selectively with bacterial species including pathogens (10) and, thus, may determine both innate and acquired immune responses to the intestinal microbiota (11, 15). There is mounting evidence that mucosal interactions between the intestinal microbiota and host immune responses partake in the development of chronic inflammatory disorder of the gastrointestinal tract (12, 16–18).

Since the first report of GP2's over-expression in the inflamed intestine of CD patients (13), several cross-sectional and prospective studies demonstrated the association of autoantibodies (autoAbs) to GP2 (aGP2) with the stricturing and/or stenosing CD phenotype and disease severity (18, 19), earlier surgical recurrence after first surgery (20), as well as de-novo development of CD in patients with suspected ulcerative colitis (UC) after ileal pouch surgery and development of subsequent pouchitis (21). Thus, autoimmunity to GP2 appears to be a stratification factor of the clinical phenotype in IBD (18).

Given the close association of PSC with IBD, the occurrence of aGP2 IgA in severe PSC (7) provided further evidence for the correlation of the mucosal loss of tolerance to GP2 with fibrostenotic changes as reported in IBD (22). Remarkably, a recent comprehensive retrospective outcome analysis of 7,121 PSC patients at 37 centers in Europe, North America, and Australia revealed that 70% of them developed IBD at some point (1). Conversely, PSC appeared to be underestimated around three-fold in long-term IBD and to progress in subclinical IBD patients (23).

In total, four human GP2 isoforms (GP2$_{1-4}$) were identified (18, 24) and respective autoAbs detected in patients with IBD which demonstrated differing test performances by enzyme-linked immunosorbent assay (ELISA) (20, 25, 26). Hence, stable HEp-2 cell-lines expressing GP2 isoforms as glycosylphosphatidylinositol (GPI)-anchored membrane molecules and one cell line with an empty vector as control were generated to elucidate the role of loss of tolerance to GP2 isoforms in PSC. Consequently, IgG and IgA aGP2$_{1-4}$ by indirect immunofluorescence assay (IFA) in patients with PSC and controls were determined.

METHODS

Patients

Patients with PSC were recruited from four European university hospitals specialized in autoimmune liver diseases (**Table 1**). All PSC patients were examined clinically and endoscopically for concomitant IBD and autoimmune hepatitis (AIH). The diagnosis of PSC and IBD was based on clinical, radiologic, endoscopic, and histologic evaluation (27–29).

In total, 145 gender-matched controls were enrolled in this study comprising 95 patients with cystic fibrosis (CF) and 50 healthy subjects (HS). The patients with CF, a multi-systemic disorder with exocrine pancreatic insufficiency and biliary cirrhosis with a different pathogenesis, were included as disease controls with regard to PSC. The 50 apparently healthy subjects (HS) with no liver or intestinal pathology were both age- and gender-matched.

The study was approved by the ethics committees of the participating centers. Written informed consent was obtained from all patients included in this study. The study was conducted in accordance with the principles of the Declaration of Helsinki (World Medical Association Declaration of Helsinki 1989).

Generation of GP2-Expressing Cell Lines

Stable HEp-2 cell lines expressing membrane GPI-anchored GP2 isoforms were generated through transduction with lentiviruses. Briefly, coding sequences of GP2 isoforms sequences were amplified with PCR and cloned into pLVX-IRES-puro plasmids each using T4 DNA ligase (ThermoFisher Scientific, Waltham, USA) in accordance with the manufacturer's protocol. To confirm successful cloning, plasmids were Sanger sequenced. For transduction, lentiviruses were produced using the Lenti-X Lentiviral Expression System (Clontech Laboratories, Mountain View, USA). Thus, an 80% confluent Lenti-X 293T cell line was co-transfected with the six plasmids containing GP2

Abbreviations: GP2, zymogen glycoprotein 2; PSC, primary sclerosing cholangitis; GP2$_1$, GP2 isoform 1; IFA, indirect immunofluorescence assay; OR, odds ratio; CI, confidence interval; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; autoAb, autoantibody; ELISA, enzyme-linked immunosorbent assay; GPI, glycosylphosphatidylinositol; AIH, autoimmune hepatitis; CF, cystic fibrosis; HS, healthy subjects; IQR, interquartile range; LTx, liver transplantation; YI, Youden index

TABLE 1 | Demographic and clinical data of patients and controls.

	n	Age (IQR)	*f* (%)	IBD (%)	CD (%)	UC (%)	AIH (%)	Cirrhosis (%)	CCa (%)	LTx (%)
PSC	212	43.0 (23.3)	70 (33.0)	136 (64.2)	17 (8.0)	119 (56.1)	20 (9.4)	86 (31.6)	5* (3.7)	81 (38.2)
Berlin	23	52.5 (17.5)	6 (26.1)	19 (82.6)§3	2 (8.7)	17 (73.9)	1 (4.3)	19 (82.6)	0	19 (82.6)
Hamburg	30	50.0 (17.3)	18 (60.0)§2	15 (50.0)	4 (13.3)	11 (36.7)§5	5 (16.7)	3 (10.0)§6	0	0
London	83	46.3 (18.7)	23 (27.7)	53 (63.9)	1 (1.2)§4	52 (62.7)	5 (6.0)	49 (59.0)§8	5 (6.0)	57 (68.8)
Debrecen	76	34.1 (21.6)§1	23 (30.3)	49 (64.5)	10 (13.2)	39 (51.3)	9 (11.8)	15 (19.7)§7	0	6 (7.9)§9
Controls	145	26.9 (22.1)&	63 (43.4)	0$	0$	0$	0$	0$	0	0
CF	95	15.6 (20.9)&	44 (46.3)$	0$	0$	0$	0$	0$	0	0
HS	50	36.0 (18.0)	19 (38.0)	0$	0$	0$	0$	0$	0	0

*AIH, autoimmune hepatitis; CCa, cholangiocarcinoma; CD, Crohn's disease; CF, cystic fibrosis; f, females; HS, healthy subjects; IQR, interquartile range; LTx, liver transplantation; n, number; PSC, primary sclerosing cholangitis; UC ulcerative colitis. *Related to 136 patients with PSC.*

Comparison of the prevalence in all patients with primary sclerosing cholangitis with control groups: &$p < 0.05$ by Kruskal-Wallis test for continuous variables.

Comparison of the prevalence in all patients with primary sclerosing cholangitis with control groups: $$p < 0.05$ by Fisher's exact test for dichotomous values.

Comparison of the prevalence within the cohorts of patients with primary sclerosing cholangitis:

§1 *Debrecen vs. Berlin, Hamburg and London, respectively ($p < 0.05$ by Kruskal–Wallis test).*

§2 *Hamburg vs. Berlin, Debrecen and London, respectively ($p < 0.05$ by Fisher's exact test).*

§3 *Berlin vs. Hamburg ($p < 0.05$ by Fisher's exact test).*

§4 *London vs. Hamburg and Debrecen, respectively ($p < 0.05$ by Fisher's exact test).*

§5 *Berlin vs. Hamburg and London, respectively ($p < 0.05$ by Fisher's exact test).*

§6 *Hamburg vs. Berlin and London, respectively ($p < 0.05$ by Fisher's exact test).*

§7 *Debrecen vs. Berlin and London, respectively ($p < 0.05$ by Fisher's exact test).*

§8 *London vs. Berlin ($p < 0.05$ by Fisher's exact test).*

§9 *Debrecen vs. Berlin and London, respectively ($p < 0.05$ by Fisher's exact test).*

isoforms or an "empty" vector. The harvested supernatants were concentrated by centrifugation in Amicon Ultra-15 centrifugal filter units (Merck Millipore, Darmstadt, Germany) and employed for transduction of HEp-2 cells. Selection of successfully transduced cells was performed by adding the antibiotic puromycin to cell culture.

Confirmation of GP2 transduction into HEp-2 cells was done with reverse transcription quantitative polymerase chain reaction (RT qPCR). In brief, RNA from transduced cells was isolated with RNeasy Mini Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). After RT reaction using Maxima First Strand Synthesis Kit (ThermoFisher Scientific), qPCR was performed in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, München, Germany) with following primers: 5- ATCAACGTGATTCCACCATCC-3 and 5- TTGAGCAAGAAGGCTGGC-3 (for GP2 gene); 5-AAATGTTTCATTGTGGGAGC-3 and 5-ATATGAGGCAGCAGTTTCTC-3 (for RPLP0 gene). RPLP0 was used as reference gene. Obtained PCR products were analyzed by electrophoresis.

Expression of GP2 isoform proteins was confirmed by Western blotting. Briefly, transduced HEp-2 cells were lysed and the lysate run on SDS-PAGE. Separated bands transferred on the blotting membrane were developed with a rabbit polyclonal antibody to GP2 reactive with all isoforms (GA Generic Assays, Dahlewitz, Germany). Isoforms of GP2 were revealed by a secondary anti-rabbit antibody conjugated with horse radish peroxidase employing enhanced chemiluminescence.

Membrane expression of GPI-anchored GP2 isoforms was confirmed by flow cytometry analysis.

Detection of IgG and IgA to GP2 Isoforms

IgG and IgA to GP2 isoforms were determined by IFA employing stably transduced HEp-2 cells expressing membrane GPI-anchored GP2 isoforms 1-4. Briefly, cells were fixed on glass slides as described elsewhere (30) and incubated with 1 in 20 diluted sera for 1 h at room temperature. HEp-2 cells transduced with an empty vector were used as negative control. After washing, bound autoAbs to GP2 isoforms were revealed by incubation of polyclonal anti-human IgG or IgA antibodies conjugated to fluorescein isothiocyanate (FITC) (Agilent, Santa Clar, USA) for 1 h at room temperature. A fluorescent microscope (Axiovert 40, Zeiss, Göttingen, Germany) was used to read specific staining of HEp-2 cells. Brighter fluorescent staining of the cellular membrane of transduced HEp-2 cells in comparison with HEp-2 cells transduced with an empty vector was scored positive.

Statistical Methods

Data were tested for normality by the Kolmogorov-Smirnov-test and non-normally distributed data were reported by median and quartile ranges. The two-tailed, Mann-Whitney and Kruskal–Wallis tests were used to test for statistically

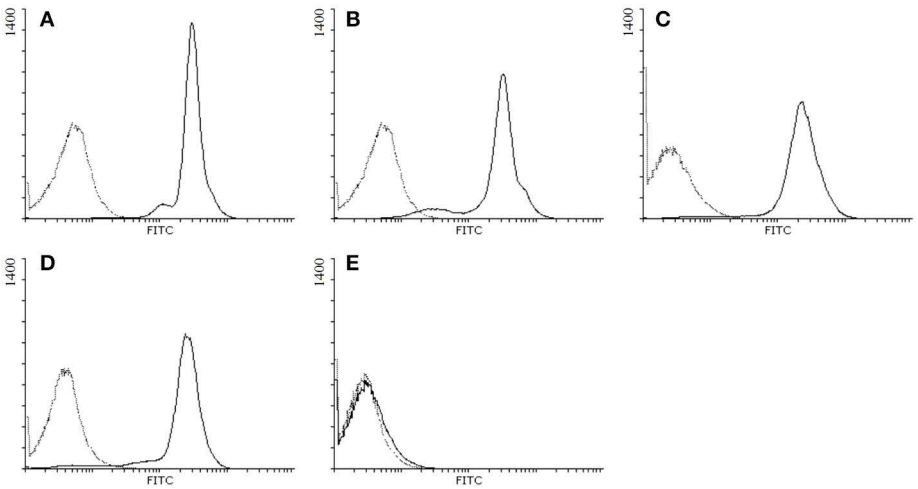

FIGURE 1 | Detection of the membrane expression of GP2 isoforms in HEp-2 cells by flow cytometry. GP2 expressed in HEp-2 cells was stained with polyclonal antibodies raised against full length human GP2 followed by FITC-conjugated anti-rabbit IgG: **(A)** HEp-2 cells expressing human GP2 isoform 1; **(B)** GP2 isoform 2; **(C)** GP2 isoform 3; **(D)** GP2 isoform 4; **(E)** HEp-2 cells transduced with an empty vector; black solid lines: primary and secondary antibody staining; black dotted lines: secondary antibody staining only.

significant differences of independent samples in 2 and more groups, respectively. Prevalence comparison between groups was performed by two-tailed Fisher's exact test. Logistic regression analysis was employed to test for the influence of explanatory (independent) variables on a binomial response variable and to detect possible clinical confounders on such association (age, gender, concomitant IBD, concomitant overlap with AIH) by a backward exclusion strategy resulting in adjusted odds ratios. $P < 0.05$ was considered as significant. MedCalc software version 12.7.0.0 (MedCalc, Mariakerke, Belgium) was used for performing statistical analysis.

RESULTS

Detection of Autoantibodies to GP2 Isoforms by Indirect Immunofluorescence

GP2 isoforms were expressed stably in HEp-2 cells as GPI-anchored molecules in the membrane of these cells by lentiviruses transduction. As control, one cell line was transduced with an empty vector only. The presence of membrane-bound $GP2_1$ to $GP2_4$ in the respective lines and their absence in the empty vector cell line was confirmed by FACS analysis (**Figure 1**).

For the detection of $aGP2_1$ to $aGP2_4$ by IFA, cells of each line were fixed to conventional glass slides and used as targets for specific autoAb analysis (**Figure 2**).

Occurrence of IgA and IgG to GP2 Isoforms in Patients and Controls

IgA and IgG against $GP2_{1-4}$ were determined in 212 patients with PSC of four European hospitals and 145 gender-matched controls. Of note, the 50 HS included as controls were gender- as well as aged-matched to all PSC patients (**Table 1**). Patients with PSC of the Debrecen cohort were significantly younger compared to the remaining three PSC cohorts whereas the Hamburg cohort had a significantly higher median age ($p < 0.05$, respectively).

Apart from $aGP2_3$, all other aGP2 demonstrated significantly elevated prevalences in PSC patients compared with controls including HS and patients with CF ($p < 0.05$, respectively) (**Table 2**). However, this did not hold true for all PSC cohorts of the four different centers.

Regarding IgA reactivity, $aGP2_1$ (47.2%) and $aGP2_4$ positivity (48.6%) revealed the highest frequencies in PSC patients resulting in an even significantly elevated combined positive rate of 66.0% ($aGP2_{1and/or4}$ IgA) compared with both rates of single aGP2 isoform IgA testing ($p < 0.0001, = 0.0004$, respectively). Analysis of all four aGP2 isoform IgA did not increase the positive rate further. Apart from $aGP2_3$ IgA, all other aGP2 isoform IgA demonstrated significantly lower prevalences in controls. Thus, $aGP2_{1and/or4}$ IgA testing revealed the best Youden index (YI) of 0.64 being a measure of assay performance.

In terms of IgG, $aGP2_1$, and $aGP2_4$ testing revealed the highest positive rates in PSC patients, too. However, their prevalences were lower in contrast to the corresponding IgA, but only the difference for $aGP2_4$ reached significance ($p = 0.0395$). Further, both aGP2 isoform IgG had significantly more positives in the control groups ($p < 0.5$, respectively). That resulted in halved YIs for both and, thus, demonstrated a poorer assay performance for the discrimination of patients with PSC from controls in comparison to corresponding IgA analysis.

Association of IgA and IgG to GP2 Isoforms With PSC Phenotypes

The possible association of the presence of IgA and IgG to $GP2_{1-4}$ in PSC patients with performed liver transplantation (LTx) and concomitant occurrence of autoimmune hepatitis, cirrhosis; cholangiocarcinoma, CD, UC, IBD (CD or UC) was

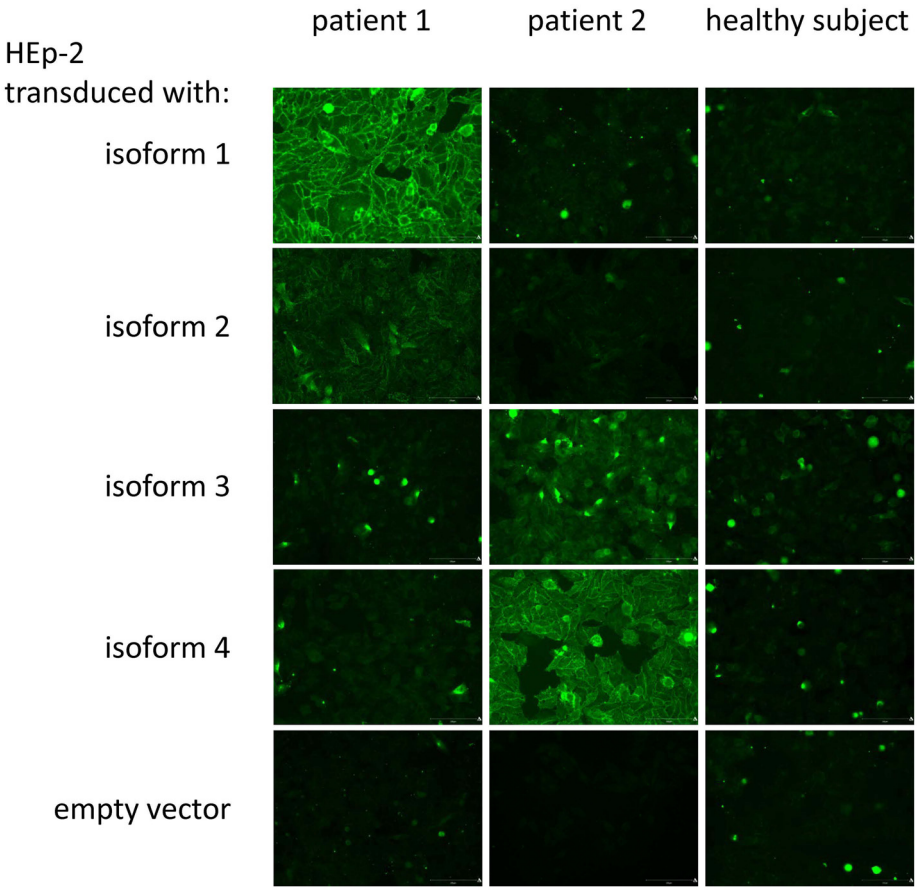

FIGURE 2 | Indirect immunofluorescence assay for the detection of IgA to GP2 isoforms: Exemplarily, two patient sera and one serum of a healthy subject as control were run on HEp-2 cells transduced with GP2 isoforms 1 ($GP2_1$) to 4 ($GP2_4$) with glycosylphosphatidylinositol anchor and an empty vector, respectively. Patient 1 demonstrated a strong specific binding to membrane-bound $GP2_1$ and a weak one to $GP2_2$, whereas patient 2 showed the typical binding pattern for a strong positive binding to $GP2_4$ and a weak one for $GP2_3$. The healthy subject did not reveal a positive membrane-reactive pattern on the respective transduced HEp-2 cells.

investigated by Fisher's exact test (**Table 3**). Further, established associations were investigated by logistic regression analysis to analyze the influence of confounding factors.

Association of IgA and IgG to GP2 Isoforms With Cirrhosis

A significantly positive association of aGP2 isoform IgA and IgG positivity in PSC was established for the concomitant occurrence of cirrhosis. Thus, $aGP2_1$ and $aGP2_4$ IgA as well as $aGP2_2$ and $aGP2_4$ IgG were more prevalent in PSC patients with cirrhosis than in those without ($p < 0.05$, respectively). Similar positive associations could be found in the larger cohorts from London and Debrecen, too. Logistic regression analysis for the risk analysis of the occurrence of cirrhosis in all 212 PSC patients confirmed $aGP2_1$ and $aGP2_4$ IgA as independent predictors in older male PSC patients (**Table 4**).

Association of $aGP2_2$ IgG With PSC Patients Without LTx

A significantly negative association of $aGP2_2$ IgA was revealed for LTx performed in PSC patients of all cohorts. This significantly more prevalent $aGP2_2$ IgA occurrence in PSC patients without LTx was not detected in the single PSC cohorts but confirmed by logistic regression analysis as independent predictor in PCS patients without LTx demonstrating concomitant cirrhosis, UC and no AIH overlap (**Table 4**).

Association of IgA and IgG to GP2 Isoforms With IBD

Patients with PSC and concomitant CD demonstrated a significantly lower prevalence of $aGP2_{1/4}$ and $aGP2_{1/2/3/4}$ IgA. However, this association was neither found in the single PSC cohorts nor confirmed by logistic regression analysis. There was no further association of all PSC patients with and without IBD, UC or CD regarding the concomitant presence of aGP2 isoform autoAbs by Fisher's exact test. Only the London cohort demonstrated significantly less frequent $aGP2_3$ IgG in PSC patients with concomitant IBD and the Hamburg cohort significantly less frequent $aGP2_2$ IgA in patients with UC ($p < 0.05$, respectively) (**Table 3**).

Logistic regression analysis revealed the concomitant occurrence of UC as an independent risk factor for liver

TABLE 2 | Frequency of IgA and IgG against GP2 isoforms 1 ($aGP2_1$) to 4 ($aGP2_4$) detected by indirect immunofluorescence assay on stabile isoform-transduced HEp2 cells in 212 patients with primary sclerosing cholangitis (PSC) from different hospitals and 145 controls.

		$aGP2_1$		$aGP2_2$		$aGP2_3$		$aGP2_4$		$aGP2_{1/2/3/4}$		$aGP2_{1/4}$	
	n	**IgA (%)**	**IgG (%)**	**IgA (%)**	**IgG (%)**	**IgA (%)**	**IgG (%)**	**IgA (%)**	**IgG (%)**	**IgA (%)**	**IgG (%)**	**IgA and/ or IgG (%)**	**IgA (%)**
PSC	212	100 (47.2)**	92 (43.4)**	21 (9.9)*	40 (18.9)*	10 (4.7)	31 (14.6)	103 (48.6)**	81 (38.2)**	140 (66.0)**	119 (56.1)**	154 (72.6)**	140 (66.0)**
Berlin	23	6 (26.1)**	7 (30.4)*	0	0	0	0	3 (13.0)*	2 (8.7)	8 (34.8)**	8 (34.8)	10 (43.5)	8 (34.8)**
Hamburg	30	11 (36.7)**	12 (40.0)*	5 (16.7)*	1 (3.3)	1 (3.3)	1 (3.3)	9 (30.0)**	8 (26.7)*	16 (53.3)**	16 (53.3)*	17 (56.7)*	16 (53.3)**
London	83	42 (50.6)**	39 (47.0)**	2 (2.4)	28 (33.7)**	4 (4.8)	26 (31.3)*	52 (62.7)**	40 (48.2)**	59 (71.1)**	50 (60.2)**	68 (81.9)**	59 (71.1)**
Debrecen	76	41 (53.9)**	34 (44.7)**	14 (18.4)**	11 (14.5)	5 (6.6)	4 (5.3)	39 (51.3)**	31 (40.8)**	57 (75.0)**	45 (59.2)**	59 (77.6)**	57 (75.0)**
Controls	145	2 (1.4)	11 (7.6)	1 (0.7)	12 (8.3)	4 (2.8)	20 (13.8)	1 (0.7)	11 (7.6)	6 (4.1)	33 (22.8)	34 (23.4)	3 (2.1)
CF	95	1 (1.1)	8 (8.4)	1 (1.1)	9 (9.5)	4 (4.2)	18 (18.9)$^{\$}$	1 (1.1)	9 (9.5)	5 (5.3)	26 (27.4)$^{\$}$	26 (27.4)	2 (2.1)
HS	50	1 (2.0)	3 (6.0)	0	3 (6.0)	0	2 (4.0)	0	2 (4.0)	1 (2.0)	7 (14.0)	8 (16.0)	1 (2.0)
YI (PSC vs. controls)		0.46	0.36	0.09	0.11	0.02	0.00	0.48	0.31	0.62	0.33	0.49	0.64

CF, cystic fibrosis, f, females; HS, healthy subjects; IQR, interquartile range; n, number; YI, Youden index (specificity+sensitivity-1)

*Comparison of the prevalence of positive aGP2 in patients with primary sclerosing cholangitis with controls (n=145): *$p < 0.05$, **$p < 0.0001$, by Fisher's exact test, respectively.*

Comparison of the prevalence of positive aGP2 in patients with cystic fibrosis with healthy subjects (n = 50): $^{\$}p < 0.05$ by Fisher's exact test.

TABLE 3 | Positive and negative (italic) significant associations of IgA and IgG against GP2 isoforms 1 ($aGP2_1$) to 4 ($aGP2_4$) with the clinical phenotype in 212 patients with primary sclerosing cholangitis (PSC) by Fisher's exact test.

	N	IBD	CD	UC	AIH	Cirrhosis	CCa	LTx
PSC	212		*$aGP2_{1/4}$ IgA p = 0.01386 $aGP2_{1/2/3/4}$ IgA p = 0.0076*			$aGP2_4$ IgA p = 0.0051 $aGP2_{1/4}$ IgA p = 0.0056 $aGP2_2$ IgG p = 0.0199 $aGP2_4$ IgG p = 0.0447	$aGP2_3$ IgG p < 0.0001	*$aGP2_2$ IgA p = 0.0006*
Berlin	23							
Hamburg	30			*$aGP2_2$ IgA p = 0.0472*				
London	83	*$aGP2_3$ IgG p = 0.0288*				$aGP2_4$ IgA p = 0.0055 $aGP2_{1/2/3/4}$ IgA p = 0.0144	$aGP2_3$ IgG p = 0.0316	
Debrecen	76					$aGP2_2$ IgA p = 0.0261 $aGP2_2$ IgG p = 0.0349		

AIH, autoimmune hepatitis; CCa, cholangiocarcinoma; CD, Crohn's disease; LTx, liver transplantation; n, number; PSC, primary sclerosing cholangitis; UC ulcerative colitis.

TABLE 4 | Logistic regression analysis of independent variables for the risk prediction of liver transplantation (LTx) and the occurrence of cirrhosis in 212 patients with primary sclerosing cholangitis (PSC).

Dependent variable	Independent variable	Coefficient	SE	OR	95% CI	*P*
cirrhosis	$aGP2_1$ IgA	0.3243	0.1514	1.3831	1.0279–1.8609	0.0322
	$aGP2_4$ IgA	0.1729	0.1771	1.5178	1.0727–2.1478	0.0185
	age	0.0273	0.0100	1.0277	1.0077–1.0480	0.0063
	gender	−0.6693	0.3235	0.5121	0.2716–0.9654	0.0385
LTx	$aGP2_2$ IgA	−3.02104	1.27092	0.0488	0.0040–0.5886	0.0175
	cirrhosis	3.70772	0.49727	40.7608	15.3800–108.0262	<0.0001
	AIH overlap	−2.63423	1.00286	0.0718	0.0101–0.5124	0.0086
	UC	1.82313	0.49012	6.1912	2.3691–16.1799	0.0002

The presence of IgA and IgG to GP2 isoforms 1 ($aGP2_1$) to 4 ($aGP2_4$) and the concomitant occurrence of inflammatory bowel disease, Crohn's disease, ulcerative colitis (UC), and autoimmune hepatitis overlap as well as age and gender were used as possible predictive independent variables for the logistic regression analysis.
CI, confidence interval, OR, adjusted odds ratio; SE, standard error.

transplantation along with cirrhosis. The presence of $GP2_2$ IgA and AIH overlap were negative predictors in this regard (**Table 4**).

DISCUSSION

The recent reports of the occurrence of aGP2 IgA in patients with large biliary duct disorders including PSC and of aGP2 IgA as marker of severe disease, as well as cholangiocarcinoma ushered in a new era in PSC serology (7, 31). Until recently, perinuclear antineutrophil cytoplasmic antibodies (ANCA) have been considered as main serological marker of PSC (32) and particularly ANCA IgA has been associated with cirrhosis linked to intestinal infections (33). However, attempts to identify the respective autoantigenic ANCA as well as other PSC-specific targets have been inconclusive so far (34). Conversely, IgG to proteinase 3 (PR3) being a cytoplasmic ANCA target could be detected by highly sensitive enzyme immunoassays or microbead-based immunoassays in up to 44% of patients with PSC (35, 36). Thus, apart from the neutrophilic target PR3, GP2 was identified as an autoantigenic target in PSC and questions on a possible pathogenic role of respective specific IgA being the mucosal immunoglobulin in contrast to IgG were raised. Since four GP2 isoforms were discovered and specific IgG and IgA against them were described in patients with IBD (24–26, 37), this study attempted to ascertain the frequency of IgG and IgA to GP2 isoforms in PSC and their possible relation to the PSC phenotype as stratification factor in PSC.

In contrast to recent reports demonstrating preferentially aGP2 IgA in PSC (7, 8), this study revealed both IgA as well as IgG against GP2 isoforms.

The IgG to GP2 isoforms determined in this study demonstrated both lower sensitivities and specificities in contrast to IgA against the corresponding GP2 isoforms which resulted

in poorer assay performances of the former. Of note, apart from significantly higher positive rates of IgG to GP2 isoforms in PSC, we detected a significantly elevated prevalence of IgG $GP2_3$Ab in control patients with CF (18.9%) vs. healthy controls (4.0%). This finding warrants further investigation and could indicate a different loss of tolerance to GP2 isoforms in CF.

In terms of discrimination of PSC from controls, $aGP2_1$ IgA (47.2%) and $aGP2_4$ IgA-positives (48.6%) revealed the highest frequencies amongst all aGP2. Interestingly, combination of both led to a significantly elevated sensitivity of 66.0%. This was a significantly higher prevalence than the corresponding total prevalence reported by Jendrek et al. (66.0 vs. 48.7%, $p < 0.0001$). Thus, combined testing of both $aGP2_1$ and $aGP2_4$ IgA appears more sensitive than the determination of IgA to just one GP2 isoform. Indeed, the single positivity rates of $aGP2_1$ and $aGP2_4$ IgA in this study were not significantly different from the rate reported elsewhere. Further, due to the higher specificity, combined $aGP2_1$ and $GP2_4$ IgA testing demonstrated the best diagnostic performance for PSC by a YI of 0.64 regarding the analysis of all aGP2 and combinations thereof.

As $GP2_1$ and $GP2_4$ represent long (537 amino acids) and short GP2 isoforms (387 amino acids), respectively, they could bear differing epitopes (24). Remarkably, $GP2_2$ and $GP2_3$ which differ from $GP2_1$ and $GP2_4$ in just three amino acids (valine-proline-arginine), respectively (25), demonstrated significantly diminished IgA binding in PSC. This adds support to the notion that GP2 isoforms bear different autoantigenic epitopes, all of which should be contained in GP2-antigenic preparations in aGP2 immunoassays for proper and accurate autoAb testing.

IgG and in particular IgA against GP2 isoforms revealed different associations with the PSC phenotype. In fact, $aGP2_1$ and $aGP2_4$ IgA were demonstrated as positive predictors of cirrhosis in older males with PSC. The cirrhosis in PSC is mainly characterized by extensive fibrosis around the larger bile ducts (4). An association of aGP2 IgG and IgA detected by ELISA with the stenosing/stricturing phenotype in patients with CD, which is characterized by fibrostenotic changes either, was established in one longitudinal and several cross-sectional studies (18). Further, the need for surgical resection and repeated surgical intervention in CD was associated with the occurrence of aGP2 (20, 22). Wölfel et al. demonstrated a significant association of $aGP2_1$ IgA and a tendency for $GP2_4$ IgA with earlier surgical recurrence in CD (20). Further, $aGP2_1$ and $aGP2_2$ IgA were reported to be significantly associated with the stenosing/stricturing and severity of disease but not with disease activity. Thus, IgA against GP2 isoforms might be a serological marker for the development of fibrostenotic changes in both the gut and larger bile ducts.

There is clearly a need for risk stratification in PSC (5, 38) and aGP2 IgA could be of prognostic value like autoAbs to gp210 do in primary biliary cholangitis (39). Given a potential ascending pathophysiology of PSC, $aGP2_{1/4}$ IgA could be a stage-defining biomarker (40). This is of interest, as aGP2 IgA secreted onto mucosal surfaces might mediate the up-take of GP2-covered bacteria by GP2-bearing M cells of the intestinal follicle-associated epithelium (15, 41) and, thus, partake in the development of severe complications or even pre-tumor stages. Patients with PSC appear to demonstrate a gut microbial signature distinct from both normal individuals and patients with UC without liver disease whereas their microbial signature is not dependent on the occurrence of IBD (42). Thus, fecal microbiota profiles can be used as markers of PSC (43). Altogether, that hints at an involvement of the microbiota in the pathophysiology of PSC which was demonstrated in a well-established mouse models for PSC and spontaneous bile duct inflammation (44, 45).

Interestingly, CD appears to confer prognostic favor in PSC and a lower risk to develop adverse effects (1). Thus, it remains to be determined in prospective studies whether aGP2-positive CD patients characterized by complicated CD with fibrotic adverse effects also demonstrate severe PSC.

All 5 PSC patients with cholangiocarcinoma demonstrated $aGP2_1$ and/or $aGP2_4$ IgA. This might be a hint that aGP2 IgA occurs earlier in the disease course and that the tolerance break is linked with the mucosal microbiota interaction of GP2. In this context, the elevation of $aGP2_3$ IgG in patients with CF is of interest since approximately 30% of patients with CF have clinically significant liver disease (46).

The finding that $aGP2_{1/4}$ and $aGP2_{1/2/3/4}$ IgA demonstrated significantly lower prevalences in PSC patients with CD was surprising. We speculate that PSC with concomitant CD might be different from CD without obvious liver involvement regarding the loss of tolerance to GP2. However, this notion needs to be treated with caution since it could be confirmed neither in the single cohorts nor by logistic regression analysis.

As most multi-center studies of this kind, our study lacks perfection. The controls were not age matched to patients with PSC due to the younger age of the controls with CF. Further, there was a high level of diversity in the distinct PSC cohorts of the four European hospitals regarding number of patients and their phenotype. The established independent predictors of cirrhosis $aGP2_1$ and $aGP2_4$ IgA could not be associated in all single cohorts with the concomitant occurrence of cirrhosis.

Altogether, combined $aGP2_1$ and $aGP2_4$ analysis is required for sensitive PSC-specific autoAb testing and should be preferred to the autoAb analysis against single aGP2 isoforms only. $aGP2_1$ and $aGP2_4$ IgA might be predictors of cirrhosis in PSC and, thus, an useful alternative for risk prediction. Elevated aGP2 IgA may demonstrate a link to fibrotic changes observed in IBD in particular in CD. Prospective studies including patients tested pre- and post-liver transplantation will provide excellent hints regarding the pathophysiological role of these autoAbs.

AUTHOR CONTRIBUTIONS

DR and PS conceived of the study and participated in its design and data evaluation. RK and JS conducted the transduction experiments and participated in the development of the indirect fluorescence assay. MS participated in the development of the indirect fluorescence assay and carried out the assays. MP, TT, DCB, JP, MM, DB, JH, ML, KC, AF, and CS recruited patients, provided patient data and participated in the statistical evaluation thereof. All authors read and approved the final manuscript.

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